

ABSTRACT

DENISE L. MATTHEWS. Studies in Chemical Toxicology: Synthesis of a Protected N²-Deoxyguanosine Adduct of a Cyclopenta-Polycyclic Aromatic Hydrocarbon. (Under the Direction of Dr. AVRAM GOLD)

A pilot synthesis was undertaken to develop synthetic routes for synthesis of N²-deoxyguanosine adducts of a cyclopenta-polycyclic aromatic hydrocarbon (CP-PAH), which have potential application for use as chromatographic standards in the ³²P-postlabeling assay. A straightforward synthetic route was developed for the synthesis of an O⁶-p-nitrophenylethyl(NPE) and 3',5'-p-toluoyl group protected derivative of 2-bromo-2'-deoxyinosine(2-BrdI) (compound 6B). The successful strategy involved sodium salt glycosylation of O⁶-NPE-protected 2-bromohypoxanthine (4) with 2-deoxy-3,5-di-O-p-toluoyl- α -D-erythro pentofuranosyl chloride (5B) to give desired pure N-9 β glycosylation product (6B) in 39% yield. The direct glycosylation product (6B) was coupled with a CP-PAH amino alcohol, trans acenaphthene 1-amino-2-ol (7), to generate the first example of a protected N²-deoxyguanosine adduct of a CP-PAH (compound 8) in ~5% yield. This successful condensation reaction proved the usefulness of the protected 2-BrdI derivative (6B) as a precursor to target N²-modified-2'-deoxyguanosine. The p-toluoyl groups can be removed by ammonolysis of compound 6B to give O⁶-NPE-protected-2-BrdI in 63% yield. The structural identity and purity of reaction products were confirmed by ¹H NMR and mass spectra.

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CHAPTER I

INTRODUCTION

Covalent interaction of chemical carcinogens with critical cellular macromolecules, especially DNA, is a key event in the overall mechanism of chemical carcinogenesis, and irreversible chemical lesions formed can initiate the carcinogenic process (Holbrook 1980; Miller and Miller 1981; Harris 1985; Williams and Weisburger 1986; Hermo 1987). Ultimate reactive and carcinogenic forms of chemicals contain an electrophilic atom capable of covalently reacting with an available nucleophilic atom of a cellular macromolecule, such as a DNA base, to form a DNA adduct (Miller and Miller 1981; Dipple, Michejda, and Weisburger 1985). Most organic genotoxic chemicals, including polycyclic aromatic hydrocarbons (PAH) and cyclopenta-PAH (CP-PAH), must undergo metabolic activation (also termed bioactivation) by cytochrome P-450 dependent mixed-function oxidases to form the electrophilic reactive intermediates (Holbrook 1980; Williams and Weisburger 1986).

Numerous studies conducted on alternant PAH, especially with benzo[a]pyrene, have determined that epoxides and dihydrodiol epoxides (especially the bay region diol-epoxide) are the reactive metabolites responsible for chemical modification of DNA and genotoxicity (see review by

Wislocki and Lu 1988). Formed by opening of the oxirane ring, carbonium ions of PAH exhibit strong regioselectivity for the exocyclic amino groups of guanine (the N-2 position) and adenine (the N-6 position) (Holbrook 1980; Jeffrey 1985; Swenberg et al. 1990).

Cyclopenta-PAH, nonalternant PAH that contain a cyclopenta ring fused to the periphery of an alternant aromatic nucleus, possess unique structures that can provide important insight into structure-activity relationships, especially the effects of molecular geometry and electronic structure on mutagenic/carcinogenic activity. Many CP-PAH are environmental contaminants formed by incomplete combustion of fossil fuels (Nesnow et al. 1986). Cyclopenta-PAH have been the focus of many recent investigations (see review by Gold, Sangaiah, and Nesnow 1988), and it has been determined that cyclopenta-ring oxidation and (if applicable) bay region diol-epoxide formation represent major pathways of metabolic activation for CP-PAH. A derivative of acenaphthylene, the smallest CP-PAH, will be utilized in this study to synthesize a deoxyguanosine adduct of a cyclopenta-PAH.

Identification and quantitation of DNA adducts in animal and human tissues, in vitro and in vivo, has become an important expanding area of research. Detection and analysis of DNA adducts can aid in formulation of the following: structure-activity relationships for mutagens/carcinogens especially in predicting genotoxic

activity of new compounds; relationships between DNA adduct formation and chemical carcinogenesis - hence, cancer risk in human populations; and relationships between levels of DNA adducts and environmental chemical exposures - hence, usefulness of DNA adducts in human biomonitoring as markers of "biologically effective dose" and as "internal dosimeters" (Wogan and Gorelick 1985; Harris 1985) of exposure to genotoxic chemicals.

Of the various immunological and physicochemical methods available to detect DNA adducts, the highly sensitive ^{32}P -postlabeling assay is well suited for aromatic adducts, and it generates autoradiograms useful as fingerprint maps for identification of unknown chemicals and mixtures (see review by Watson 1987).

Using ^{32}P -postlabeling techniques, partial structural elucidation of DNA adducts (base/metabolite composition) has been accomplished by comparing autoradiograms from DNA of C3H10T1/2 cells exposed to CP-PAH with autoradiograms from homo oligodeoxynucleotides treated with chemically synthesized ultimate active metabolites of CP-PAH (Nesnow et al. 1989; Lasley et al. 1990). For detailed structural characterization of adducts, co-chromatography of chemical synthetic standards of known structure (modified deoxynucleosides/deoxynucleotides) with cellular adducts is necessary. This type of identification procedure has been reported in the literature for styrene oxide-DNA adducts (Pongracz et al. 1989). Detailed structural elucidation of

DNA adducts, which will identify specific sites of attack on the DNA bases, will provide valuable information at the molecular level about the initial interactions between chemical mutagens/carcinogens and DNA. Based on ^{32}P -postlabeling results for aceanthrylene (Nesnow et al. 1989) and benz(j)aceanthrylene (Lasley et al. 1990) and the documented regioselectivity of PAH towards the exocyclic amino group of guanine, initial synthetic targets should be cyclopenta ring and bay region diol-epoxide adducts at N^2 of deoxyguanosine.

Cyclopenta-PAH amino alcohols of acenaphthylene and aceanthrylene have been successfully synthesized and used to generate N-6 adenosine adducts (RNA adducts) expected from attack of the corresponding ring-opened cyclopenta epoxides (Bartczak et al. 1989). Later these amino alcohol derivatives were successfully condensed with 6-chloropurine-9-(2-deoxy- β -D-ribose) to produce N^6 -deoxyadenosine adducts (DNA adducts). Although N^2 -aryl and N^2 -alkyl 2'-deoxyguanosine derivatives have been reported in the literature (Wright and Dudycz 1984; Casale and McLaughline 1990; Lee et al. 1990; Hildebrand et al. 1990), no N^2 -deoxyguanosine adduct of a cyclopenta-PAH has been synthesized.

Thus, this pilot study was undertaken to develop synthetic routes for synthesis of N^2 -modified deoxyguanosine using the CP-PAH amino alcohol derivatives previously prepared, specifically trans acenaphthene 1-amino-2-ol. The

appropriate halopurine precursor to an N²-deoxyguanosine adduct is a 2-halo-2'-deoxyinosine; however, purine derivatives of this type are not commercially available. Specific objectives of this study included the following:

1) Develop a straightforward synthetic route for generation of 2-bromo-2'-deoxyinosine (2-BrdI) or a suitably protected derivative. This was to be accomplished by:

A) Synthesis of a protected 2-bromohypoxanthine derivative.

B) Glycosylation of above compound with some derivative of 2'-deoxyribose with the objective of obtaining the N-9 β -substituted isomer as predominant, isolable glycosylation product.

2) Test applicability of 2-bromo-2'-deoxyinosine or a derivative as a precursor to N²-deoxyguanosine adducts of a cyclopenta-PAH by using it to synthesize a (protected) N²-modified deoxyguanosine:

A) Carry out condensation reaction(s) between 2-BrdI or its derivative and trans acenaphthene 1-amino-2-ol.

3) Evaluate results of the synthetic strategy employed in this pilot study and make recommendations that will facilitate the future synthesis of biologically significant CP-PAH - deoxyguanosine adducts in quantity for eventual application as chromatographic standards in the ³²P-postlabeling assay.

CHAPTER II

LITERATURE REVIEW

General Mechanism of Chemical Carcinogenesis

Environmental factors, including certain radiations, specific viruses, some chemicals, and combinations of the above, play a strong role in determining the occurrence of many human cancers (Miller and Miller 1981). Of the six million or so chemical compounds which have been identified and catalogued, about 35 have been recognized by the International Agency for Research on Cancer (IARC) to be causally associated with increased risk of human cancer (IARC 1987). Despite the limited number of compounds which are recognized as human carcinogens, the list can be expanded if animal test data is included. Also, many more chemical compounds have been found to transform or mutate cells in culture.

Currently, it is well accepted that chemical carcinogenesis is a complex multistage process involving initiation, promotion (including tumor conversion), and progression, as shown in figure 1 (Holbrook 1980; Miller and Miller 1981; Harris 1985; Williams and Weisburger 1986; Herms 1987). Chemicals play key roles as initiating carcinogens and promoting agents in this scheme. Interaction of chemical carcinogens with critical cellular

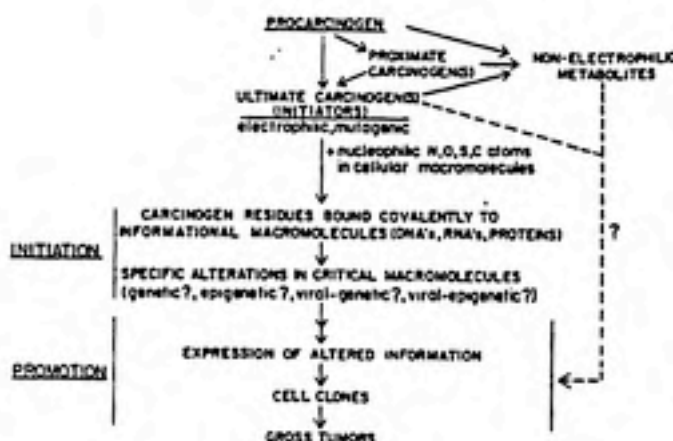
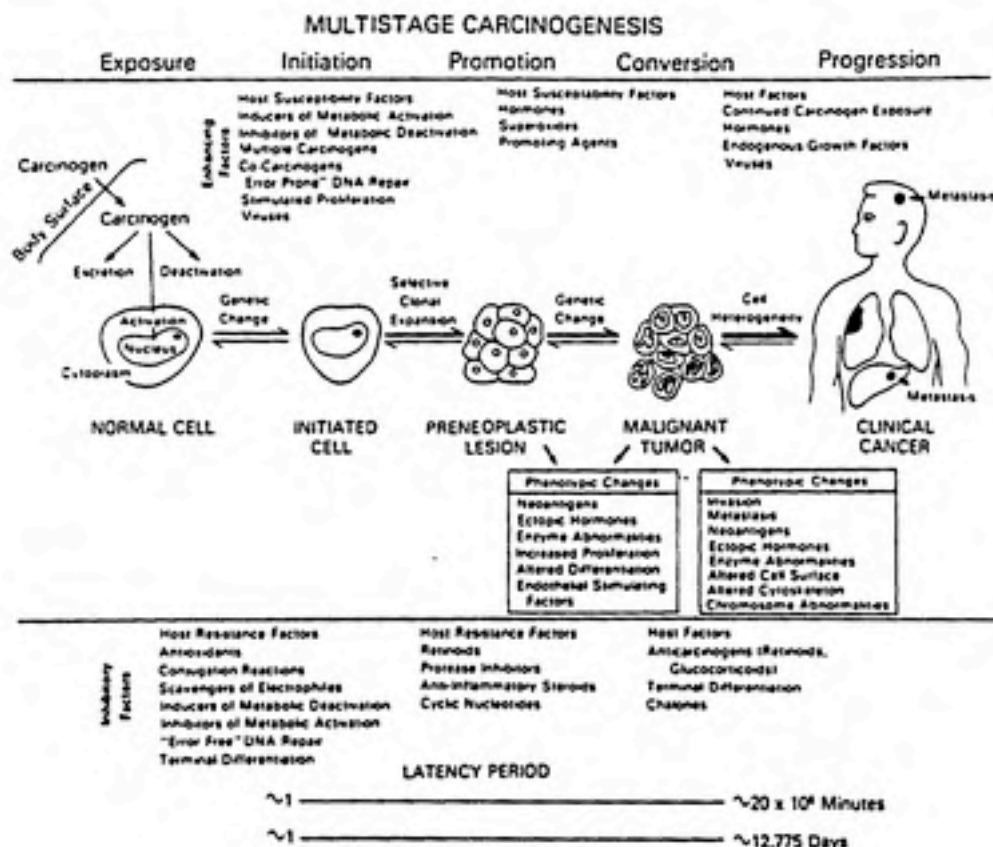


Fig. 1. The Multistage Process of Chemical Carcinogenesis. Top, from Harris 1985, p. 186; Bottom, from Miller and Miller 1981, p. 2338.

macromolecules leading to covalent adducts, especially of DNA, is a significant event in the overall mechanism of chemical carcinogenesis, and irreversible chemical lesions formed can initiate the carcinogenic process. Such genetic events followed by cell proliferation may lead ultimately to the induction of neoplasia, a process that can span twenty years or longer in humans.

Hermo (1987) describes the series of events that are generally referred to as "initiation". These include: (1) absorption, distribution, and possible biotransformation (metabolic activation) of a chemical; (2) interaction of an electrophilic ultimate carcinogenic metabolite with cellular macromolecules, particularly DNA (but also RNA/proteins); and (3) the fixation of carcinogen damage through cell replication producing a permanent genetic change.

The ability of chemical carcinogens to covalently bind to and damage DNA can lead to cellular mutational events, such as point mutations and frame-shift mutations, as well as other types of gross structural DNA alterations like gene rearrangement and amplification (Hermo 1987). Codon rearrangement may involve sequences known as oncogenes, and several mechanisms, including point mutation, have been identified as being responsible for the activation of cellular proto-oncogenes (Williams and Weisburger 1986). Gene products of many oncogenes function in biochemical processes that could be involved in malignant cell transformation (Marx 1989).

Thus, carcinogen-DNA adduct formation can produce a permanently altered or "initiated" cell (also known as a latent preneoplastic cell) which may, in the future, undergo cell divisions and changes in gene expression ultimately leading to production of a neoplasm.

The "promotion" stage of carcinogenesis involves the conversion of an initiated precancerous cell to the transformed neoplastic state then capable of proliferation leading to clonal amplification and malignant tumor formation (Herms 1987). Cells that have undergone neoplastic conversion may remain dormant, held in check by tissue homeostatic factors, but their proliferation may be facilitated by various chemical promoting agents, such as phorbol esters (Williams and Weisburger 1986). Harris (1985) mentions the concept of tumor conversion, whereby benign tumors can convert to malignant tumors, and he suggests that DNA-damaging and mutagenic chemical agents may cause an additional genetic event that hastens this conversion.

Many of the specific steps in chemical carcinogenesis, such as bioactivation and DNA repair, are controlled and modified by endogenous and exogenous host factors including: species; sex; age; and immunological, hormonal, and nutritional conditions (Williams and Weisburger 1986).

Besides the genetic mechanism described above, there is an alternative, perhaps accompanying, mechanism for permanent neoplastic conversion that involves epigenetic

effects on gene expression, possibly stemming from carcinogenic interaction with proteins and/or RNA (Holbrook 1980; Williams and Weisburger 1986). As discussed by Williams and Weisburger (1986), this has led to the attempted classification of chemical carcinogens as genotoxic if they interact with and alter DNA or as epigenetic if a biologic effect other than reaction with DNA could be the basis for their carcinogenicity.

Metabolism of Xenobiotics in Relation to
DNA Adduct Formation

Many studies on a variety of chemical carcinogens have shown that their ultimate reactive and carcinogenic forms contain an electrophilic (relatively electron-deficient) atom capable of reacting nonenzymatically by covalent bond formation with an available nucleophilic (electron-rich) atom of a critical cellular macromolecule (DNA, RNA, and/or protein) (Miller and Miller 1981; Dipple, Michejda, and Weisburger 1985). Direct-acting or primary genotoxic carcinogens contain intrinsically electrophilic centers and do not require metabolic activation. They include such chemicals as: alkyl imines, lactones, alkylene epoxides, sulfate esters, mustards, halo ethers, and certain nitrosamides (Holbrook 1980; Williams and Weisburger 1986).

In contrast, most organic genotoxic carcinogens are indirect-acting or secondary carcinogens. They are not reactive as the parent compound (termed "pro-" or "precarcinogen") and must undergo enzymatic metabolic

activation (or bioactivation) to the reactive electrophilic "ultimate carcinogen" (Holbrook 1980; Hermo 1987). The term "proximate carcinogen" applies to any intermediate species involved in the overall metabolic activation. Indirect-acting chemicals include: polycyclic aromatic hydrocarbons, aromatic amines, quinolines, nitrofurans, nitrosamines, azo compounds, and more (Williams and Weisburger 1986).

The enzymes necessary for metabolic activation of xenobiotics (chemicals entering an organism from the external environment) are concentrated primarily in the endoplasmic reticulum of hepatocytes. The primary role of these systems is the biotransformation and detoxication of lipophilic compounds to more water-soluble hydrophilic species which can be more readily excreted from the organism. Sipes and Gandolfi (1986) and Singer and Grunberger (1983) discuss the role of hepatic systems with particular regard to activation of promutagens and procarcinogens.

The enzyme-dependent reactions are divided into two stages: Phase I, such as oxidation, reduction, and hydrolysis reactions in which substrates are functionalized to increase hydrophilicity; and Phase II conjugation reactions in which a highly polar endogenous group is added to the hydrophilic site generated by Phase I action. Of most importance in metabolic activation are the Phase I oxidative enzymes in the cytochrome P-450 system, otherwise

known as the mixed function oxygenase (MFO) system, monooxygenases, or mixed-function oxidases.

For example, in the case of polycyclic aromatic hydrocarbons or olefinic compounds, one oxidation pathway involves the cytochrome P-450-mediated addition of oxygen to the carbon-carbon double bond to produce an epoxide. Containing a highly strained 3-membered ring, epoxides are very reactive intermediates capable of opening to an electrophilic carbonium ion which can form covalent adducts with cellular nucleophiles including the DNA bases. Epoxide hydrolase (formerly epoxide hydrase/hydratase) is an important hydrolytic enzyme that can deactivate epoxides by catalyzing the hydration of arene oxides and aliphatic epoxides to their corresponding trans-dihydrodiols, which can be conjugated and excreted. Some aromatic dihydrodiols, however, can undergo secondary metabolism by the MFO enzymes, giving diol-epoxides which may be potent mutagens and carcinogens. This pathway has been identified as the major activation route of benzo[a]pyrene.

Thus, the same enzymes that biotransform and detoxify xenobiotics to protect the body can also produce reactive intermediates (including proximate and ultimate carcinogens) which are inherently capable of causing more harm than the original parent chemical compound that entered the body. The balance between rates of formation and rates of detoxication of these reactive metabolites is important in

determining whether adverse cellular events might later occur.

Metabolic Activation of Alternant PAH

Polycyclic (or polynuclear) aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants formed from the incomplete combustion of fossil fuels and have been identified in gasoline and diesel fuel exhaust, coke oven emissions, coal soot, coal tar and its pitches, mineral oils, tobacco smoke, and charred food. As potential mutagens, carcinogens, and teratogens, PAH pose human health hazards due to public exposure to these compounds through inhalation of contaminated air or consumption of certain food products (Sawicki 1985; Boulos and von Smolinski 1986).

During the past two decades, the most commonly and extensively studied subset of PAH has been the "alternant" structures composed of fused aromatic 6-membered rings. "Nonalternant" PAH such as cyclopenta-PAH are characterized by a cyclopenta ring fused to the periphery of an alternant aromatic nucleus. They possess unique structures that can provide important insight into structure-activity relationships. In order to understand the metabolic activation pathways of cyclopenta-PAH, it is important to consider the results of previous studies on alternant PAH, especially those supporting the role of epoxides and diol-epoxides as the reactive metabolites responsible for chemical modification of DNA and genotoxicity.

As described by Dipple, Michejda, and Weisburger (1985) and shown in figure 2(A), oxidation of a PAH double bond catalyzed by the cytochrome P-450-dependent arylhydrocarbon hydroxylases (AHH) can produce an arene oxide. There are three known possible outcomes for this initially formed epoxide: nonenzymatic rearrangement to a phenol; hydration catalyzed by the epoxide hydrolase enzyme to a trans-dihydrodiol; or glutathione conjugation catalyzed by the glutathione-S-transferases leading to mercapturic acid excretion products. One of these three routes can usually inactivate most primary epoxide metabolites of PAH, offering no chance for epoxide ring opening to a benzylic carbonium ion capable of reacting with critical cellular macromolecules.

For many years the simple "K-region" epoxide was proposed as the metabolically formed DNA-reactive metabolite and the ultimate carcinogen of many PAH. Beginning in the 1970s, numerous studies on the metabolic activation of PAH focused on benzo[a]pyrene, abbreviated B[a]P (see review by Wislocki and Lu 1988). These investigations led to the discovery of the vicinal dihydrodiol epoxide as the ultimate carcinogen, formed by further enzyme-dependent oxidation of the isolated double bond adjacent to a trans-dihydrodiol. Most of the biologically active diol-epoxides were formed on a 3-sided peripheral indentation called the "bay region". Figure 2(B) outlines the metabolic reaction scheme for a PAH from the parent compound through the dihydrodiol as the

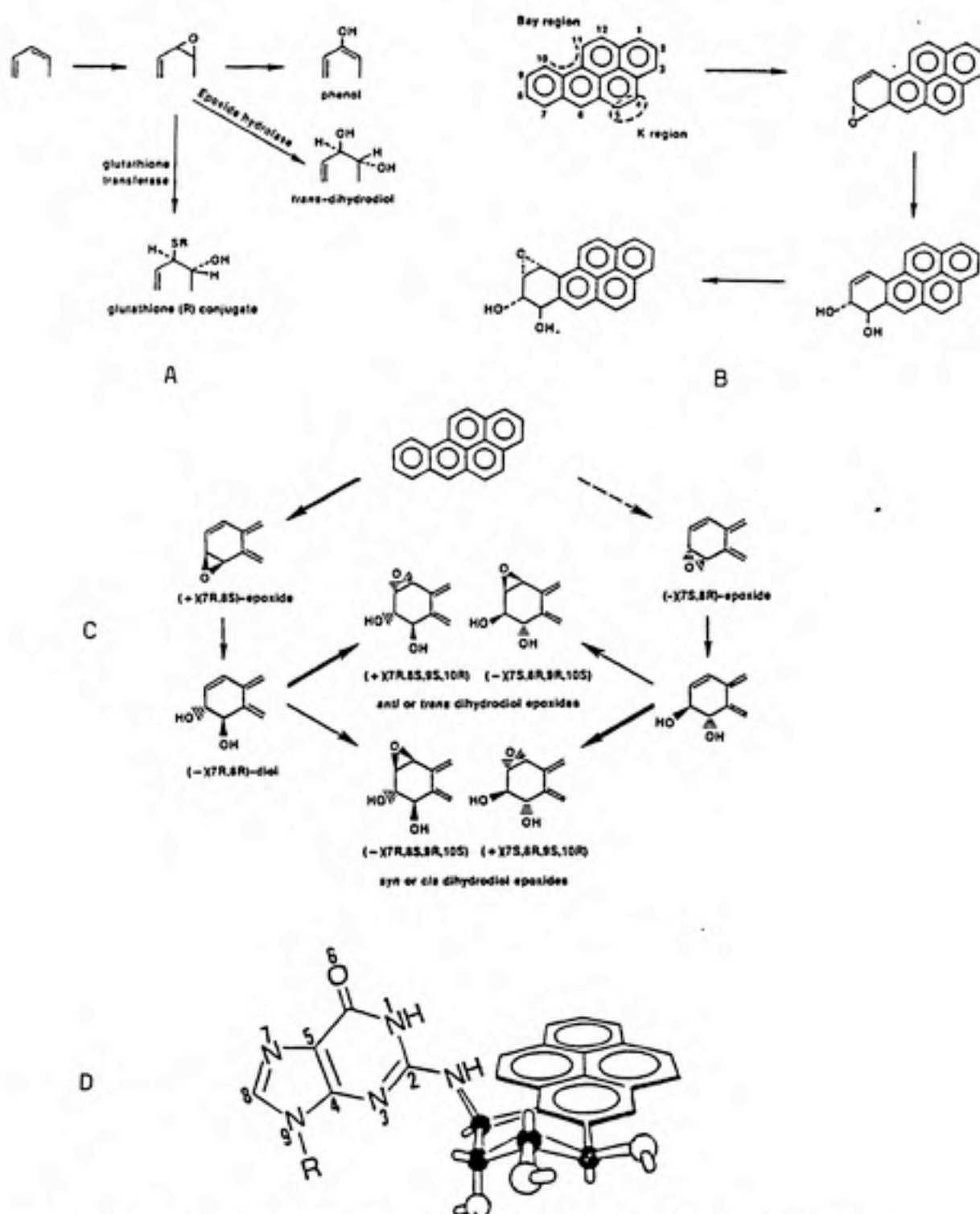


Fig. 2. Metabolic Activation of a Polycyclic Aromatic Hydrocarbon. (A), Disposition of an Epoxide (from Dipple, Michejda, and Weisburger 1985, p. 227); (B), Formation of a Bay Region Diol-Epoxy, (same ref., p. 274); (C), Stereoisomers from Metabolism of B(a)P (same ref., p. 275); (D), DNA Adduct of B(a)P (from Herms 1987, p.10).

proximate carcinogen to the bay region diol-epoxide as the ultimate carcinogen.

As discussed by Grover (1982), the metabolic conversion of dihydrodiols to diol-epoxides may depend on whether the conformation of the dihydrodiol hydroxyl groups is diaxial (approximately perpendicular to the plane of the molecule) or diequatorial (approximately within the same plane as the plane of the molecule). When the diol groups are adjacent to a bay in the molecule or an alkyl substituent, they may be forced to adopt a quasi-diaxial conformation. In this conformation further metabolism to the diol-epoxide does not readily occur possibly because of stereochemical effects preventing the enzyme-substrate interaction. However, when an unhindered dihydrodiol adopts the quasi-diequatorial conformation, as it does for the precursor to the bay region diol-epoxide in which the epoxide is adjacent to the bay, further oxidative metabolism can and does take place.

According to in vitro studies, bay region diol-epoxides can be converted to glutathione conjugates but do not act as substrates for epoxide hydrolase, an important factor in determining their mutagenic and carcinogenic capabilities (Grover 1982; Dipple, Michejda, and Weisburger 1985).

There are four possible stereochemical isomers of the bay region diol-epoxides for benzo[a]pyrene (see figure 2(C)), all of which have been chemically prepared. Use of these isomeric derivatives as reference compounds has shown that both metabolic formation and reaction with DNA and

biological activity are highly stereoselective with (+)-anti-7,8-dihydrodiol-9,10-epoxide determined to be the most carcinogenic ultimate species (Jeffrey 1985; Dipple, Michejda, and Weisburger 1985; Wislocki and Lu 1988).

Figure 2(D) shows the structure of the trans B[a]P-DNA adduct expected upon epoxide ring opening of the ultimate carcinogenic species to form an electrophilic carbonium ion at C-10 that covalently binds to the exocyclic amino group of deoxyguanosine. Because of the high stability of the C-10 carbonium ion, a mixture of cis and trans adducts would be predicted, but the trans isomer represents greater than 90% of the DNA adducts (Jeffrey 1985).

In 1976, Jerina et al. proposed the "bay region theory" to explain the high biological activity of bay region diol-epoxides (see reviews by Wood et al. 1979; Lehr et al. 1982; Jeffrey 1985; and Wislocki and Lu 1988). The concept postulates that epoxides situated on saturated, angular benzo-rings located in the bay region of a PAH (bay region diol-epoxides) should be highly reactive due to greater ease of electrophilic carbonium ion formation. This prediction is based upon Dewar perturbational molecular orbital (PMO) calculations which estimate resonance stabilization energies ($\Delta E_{deloc} / \beta$ values) of benzylic carbonium ions formed upon ring opening of the epoxides. Based upon studies on alternant bay-region PAH, larger values of $\Delta E_{deloc} / \beta$ correspond to greater stabilization of the oxirane ring-opened carbocation and, in general, to greater biological

activity of the metabolite with a value of $\Delta E_{deloc} / \beta > 0.7$ determined to be a possible threshold for carcinogenic activity (Jerina et al. 1976; Wood et al. 1979; Lehr et al. 1982).

Besides B[a]P, the bay region diol-epoxides of other PAH including benz[a]anthracene, dibenz[a,h]anthracene, 7,12-dimethylbenz[a]anthracene, and 3-methylcholanthrene have proven to be the most chemically reactive forms, exhibiting higher mutagenic/carcinogenic activity than metabolites at non-bay region molecular sites (Grover 1982; Singer and Grunberger 1983).

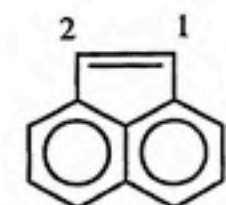
The bay region theory predicts chemical reactivities of the highly stabilized oxirane ring-opened benzylic carbonium ions formed from bay region diol-epoxides solely on the basis of perturbational delocalization energies. It does not account for regioselectivity of metabolism, stereochemical, conformational, and/or structural parameters (like molecular size), which can all influence the interaction of the reactive intermediate species with critical cellular nucleophiles.

Metabolic Activation of Cyclopenta-PAH

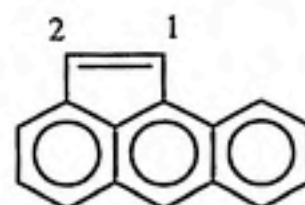
Cyclopenta-polycyclic aromatic hydrocarbons (CP-PAH) are characterized by the fusion of an ethylene fragment to an alternant PAH molecule to form a new PAH containing an unsaturated five-membered ring (Nesnow et al. 1986). This unique class of nonalternant PAH offers further

opportunities to investigate the roles of molecular geometry, stereochemistry, and electronic structure in mutagenic and carcinogenic activity, especially for comparison with predictions based upon previous studies of alternant PAH. In the past decade CP-PAH studies have focused on the importance of cyclopenta ring and bay region (if present) features in the mixed-function oxidase activation of these compounds as well as the correlation of PMO resonance stabilization energy ($\Delta E_{deloc} / \beta$) calculations with biological activity (see reviews by Nesnow et al. 1986; Gold, Sangaiah, and Nesnow 1988).

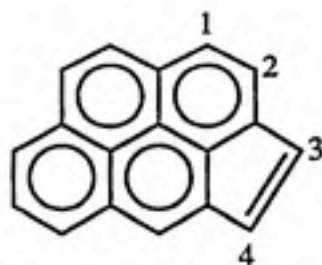
Figure 3 depicts the molecular structures, names, and common abbreviations of eight cyclopenta-PAH which have been well studied for genotoxic activity. Of these CP-PAH, at least five have been identified as environmental contaminants primarily from combustion processes, as indicated in table 1. The smallest cyclopenta-PAH, acenaphthylene, contains three rings and is also an environmental contaminant but is not a bacterial mutagen (Kaden, Hites, and Thilly 1979).



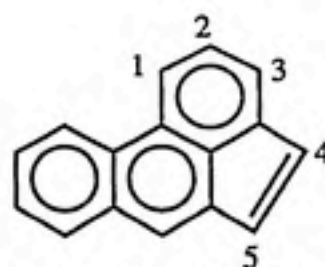
Acenaphthylene
AN



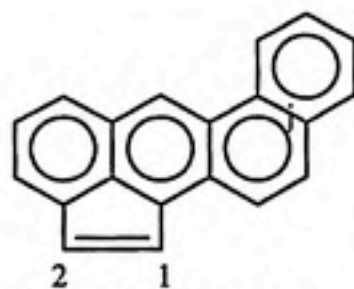
Aceanthrylene
ACE or AA



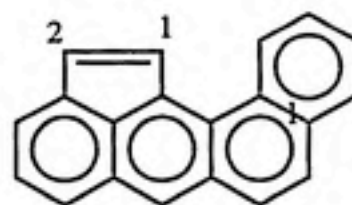
Cyclopenta[cd]pyrene
CPP



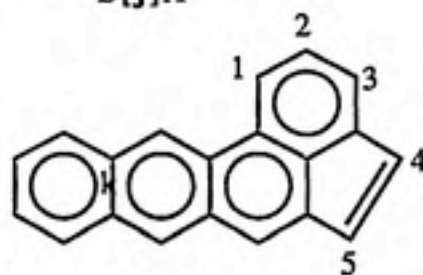
Acephenanthrylene
AP



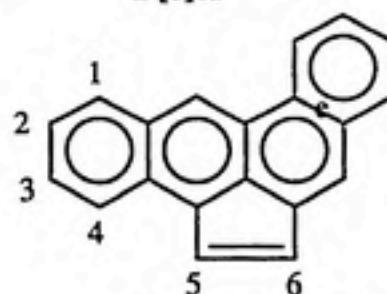
Benz[j]aceanthrylene
B[j]A



Benz[l]aceanthrylene
B[l]A



Benz[k]acephenanthrylene
B[k]A



Benz[e]aceanthrylene
B[e]A

Fig. 3. Structures of Some Cyclopenta-PAH Studied for Genotoxic Activity.
(modified from Nesnow et al. 1986, p. 516)

Table 1. -- Environmental Occurrence of Cyclopenta-PAH

Parent PAH	CP-PAH	Sources
Pyrene	CPP	Automobile exhausts Coal combustion Wood combustion Tobacco smoke Carbon black Pitch
Phenanthrene	AP	Grilled foods Coal combustion Kerosene combustion Wood smoke Tobacco smoke Carbon black
Benz[a]anthracene	B[e]A B[j]A B[l]A	Coal combustion Coal combustion Wood smoke

Source: modified from Nesnow et al. 1986, p. 517; Krishnan and Hites 1981, p. 342.

In 1980 Fu, Beland, and Yang presented PMO calculations on the presumed ultimate carcinogenic metabolites, the cyclopenta-PAH epoxides, for over fifty CP-PAH compounds. They reported that the olefinic double bond of the fused five-membered ring in most CP-PAH has the highest bond order making it highly susceptible to epoxidation and that the benzylic carbonium ion resulting from ring opening of the cyclopenta epoxide may be effectively stabilized by the aromatic system. In fact, 33 of the CP-PAH epoxides can yield carbonium ion intermediates having higher resonance stabilization/delocalization energies ($\Delta E_{deloc}/\beta$) than that of the mutagenic and carcinogenic bay region diol-epoxide of B[a]P (7,8-dihydrodiol-9,10-epoxide; $\Delta E_{deloc}/\beta = 0.794$). Fu and co-workers (1980) proposed that some

cyclopenta-PAHs may present a mutagenic and/or carcinogenic hazard similar to the classic alternant PAH since the five-membered ring of CP-PAH provides a site for metabolic activation by mixed-function oxidases.

Around the same time period, Eisenstadt and Gold (1978) and Gold et al. (1980) reported their studies on the metabolic activation, mutagenesis, and morphological transforming capability of the non-bay-region cyclopenta[cd]pyrene (CPP) and its cyclopenta epoxide, the 3,4-oxide. They found CPP to be highly mutagenic with rat liver S9 activation in the Ames assay, CPP 3,4-oxide to be a direct-acting mutagen in bacterial and mammalian assays, and both compounds able to transform mammalian cells in culture. Gold and co-workers suggested that CPP 3,4-oxide might be the ultimate mutagenic form of the parent CP-PAH, and this conclusion was further supported by identification of the dihydrodiol, 3,4-dihydroxy-3,4-dihydro-cyclopenta[cd]pyrene, as the major metabolite in bacterial and C3H10T1/2 mouse cells (Gold, Schultz, and Eisenstadt 1979; Nesnow et al. 1981).

Of six other CP-PAH studied, including aceanthrylene, acephenanthrylene, B[e]A, B[j]A, B[l]A, and B[k]A, liver microsomes from Aroclor-1254 induced rats metabolized all compounds to cyclopenta-ring dihydrodiols, implicating epoxide intermediates (Sangaiah et al. 1983, 1986; Nesnow et al. 1984). Using the same rat liver S9 activation system, all of the above CP-PAH were found to be mutagenic in the S.

typhimurium Ames assay (Nesnow et al. 1984; Sangaiah et al. 1983, 1986). The four cyclopenta-fused isomers of benz[a]anthracene were also mutagenic in mammalian cells in culture (Nesnow et al. 1984).

In S. typhimurium the peak mutagenic activity of B[j]A, B[e]A, and B[l]A occurred at considerably lower S9 concentrations than B[a]P and B[k]A (Nesnow et al. 1984). Low optimum S9 concentration has been reported previously for CPP and was interpreted as indicating activation via a one-step cyclopenta-ring epoxidation as opposed to the multistep diol-epoxide activation pathway of B[a]P (Eisenstadt and Gold 1978).

As shown in table 2, the delocalization energies for oxirane ring-derived carbocations on the cyclopenta rings of B[j]A, B[e]A, and B[l]A exceed that of the mutagenic and carcinogenic bay region C-10 carbonium ion of B[a]P-7,8-dihydrodiol-9,10-epoxide.

Table 2. -- PMO Delocalization Energies of Benzylic Carbonium Ions Derived from Epoxides of PAH

Epoxide	Carbonium ion	$\Delta E_{deloc} / B$
ACE-1,2-oxide	C-1	0.931
B[j]A-1,2-oxide	C-1	0.879
B[e]A-5,6-oxide	C-5	0.879
B[l]A-1,2-oxide	C-1	0.833
B[a]P-7,8-diol-9,10-oxide	C-10	0.794
CPP-3,4-oxide	C-3	0.794
B[k]A-4,5-oxide	C-5	0.722
AP-4,5-oxide	C-5	0.664

Source: modified from Bartczak 1988, p. 80.

The cyclopenta-ring arene oxides of B[j]A, B[e]A, B[l]A, and B[k]A were synthesized and found to be direct-acting mutagens in the Ames assay with B[k]A oxide the least potent in agreement with its lower $\Delta E_{deloc} / \beta$ value (Bartczak et al. 1987, 1988). The results of this study support the hypothesis that the cyclopenta epoxides of the three benzaceanthrylene isomers B[e]A, B[j]A, and B[l]A are major contributors to mutagenicity in the Ames assay, but other metabolic activation pathways may play a role in the mutagenicity of B[k]A. They also reaffirm the utility of the $\Delta E_{deloc} / \beta$ parameter as a predictor of mutagenic activity.

Three of the four cyclopenta-benz[a]anthracene isomers (B[e]A, B[j]A, and B[l]A) were active in morphologically transforming C3H10T1/2 cells (Mohapatra et al. 1987). In this assay B[j]A and B[l]A were metabolized to cyclopenta-ring dihydrodiols as well as K-region dihydrodiols and the dihydrodiol precursor to the bay region diol-epoxide as major metabolite for B[j]A. These findings suggest that alternative routes of metabolic activation besides cyclopenta-ring oxidation, such as K-region or bay region activation, may be operative in C3H10T1/2 cells.

Recently, the cell transforming activities of B[j]A, its cyclopenta epoxide, cyclopenta dihydrodiol, 9,10-dihydrodiol, and bay region diol-epoxide were studied. Results establish that both cyclopenta-ring epoxidation and

formation of the bay region diol-epoxide represent major independent pathways of metabolic activation (Lasley et al. 1990).

Gold, Sangaiah, and Nesnow (1988) provide a detailed account of many CP-PAH studies including bioassay results, metabolic characterization, and structure-activity considerations.

Molecular Sites on DNA Susceptible to Covalent Reactions

The covalent reactions of electrophilic ultimate mutagens/carcinogens with susceptible nucleophilic centers in DNA can lead to a wide variety of DNA adducts. Which nucleophilic molecular sites on DNA are attacked depends upon several factors including the reaction mechanism (as unimolecular S_N1 or bimolecular S_N2), the strength of the nucleophile, characteristics of the electrophile such as hardness and stability, and steric constraints (Jeffrey 1985; Swenberg et al. 1990). The most strongly nucleophilic sites in DNA involve the ring-nitrogens, particularly N-7 of guanine, and the more weakly nucleophilic sites include the oxo and amino groups of the four DNA bases (Holbrook 1980; Jeffrey 1985; Swenberg et al. 1990).

Alkylating agents, such as methyl methanesulfonate, that react by the S_N2 mechanism favor the most nucleophilic sites, like the N-7 position of guanine, in the absence of steric factors (Holbrook 1980; Jeffrey 1985; Swenberg et al. 1990). Chemical agents, such as methylnitrosourea, that

react by the S_N1 mechanism tend to be less selective and react efficiently at oxygens and other nitrogens in DNA. This can lead to alkylation on positions such as: N-1, N-3, and N-7 of adenine; N-3 and O⁶ of guanine; N-3 of cytosine; O⁴ of thymine; and the hydroxyl of phosphates (Holbrook 1980; Jeffrey 1985; Swenberg et al. 1990).

Proceeding by the S_N1 mechanism, metabolites of aromatic amines and amides react preferentially with C-8 of guanine. N² of guanine and N⁶ of adenine are frequent positions of attack by the stable carbonium ions of polycyclic aromatic hydrocarbons (Holbrook 1980; Jeffrey 1985; Swenberg et al. 1990).

Adducts formed at several sites that normally participate in the hydrogen bonding of complementary bases (such as N-3 of cytosine, O⁶ of guanine, or O⁴ of thymine) are critical with respect to mutagenesis and carcinogenesis because they have the potential to induce base mispairing when DNA is replicated (Holbrook 1980; Herms 1987; Swenberg et al. 1990).

Methods of Identification and Quantitation of DNA Adducts

Detection and characterization of DNA adducts can aid in the formulation of structure-activity relationships for mutagens/carcinogens (including stereo- and regioselectivity factors) and can also help establish relationships between DNA adducts and exposure to environmental chemicals as well as initiation of chemical carcinogenesis. There are

currently several immunological and physical techniques available to identify and measure adducts in DNA isolated from exposed tissues and cells of animals and humans, both in vitro and in vivo (see table 3). Wogan and Gorelick (1985), Farmer, Neumann, and Henschler (1987), and Swenberg et al. (1990) provide overviews of the various methods.

Table 3. -- Physical and Immunological Methods to Identify DNA Adducts

Assays	Sensitivity	DNA used
HPLC - fluorescence detection	1 adduct/ 10^7 bases	100 ug
HPLC - UV	2 adducts/ 10^5 bases	2600 ug
Synchronous fluorescence spectrophotometry(SFS)	1 adduct/ 10^7 bases	---
Radioimmunoassay(RIA)	1 adduct/ 10^5 bases	1 ug
Enzyme radioimmunoassay (competitive USERIA)	1 adduct/ 10^{7-8} bases	25 ug
^{32}P -postlabeling	1 adduct/ 10^{7-10} bases	1 ug
1 adduct/diploid mammalian genome = 1 / 1.2×10^{10} bases		

Source: modified from Wogan and Gorelick 1985, p. 12; Farmer, Neumann, and Henschler 1987, p. 256.

Much knowledge on the interactions between carcinogens and DNA has been obtained by the reaction of radiolabeled chemicals with DNA or their administration to cells or animals followed by separation, quantification, and identification of the adducts. However, this approach is limited by the small number of compounds available in radiolabeled form, the high cost of synthesis, the rather

"large" amounts (up to mg) of DNA required for analysis, and inapplicability to studies in humans.

High pressure liquid chromatography (HPLC) in conjunction with UV or fluorescence detection has been employed for detection of B[a]P-DNA adducts. Synchronous fluorescence spectrophotometry (SFS) can be useful for carcinogens that fluoresce, like PAH and aflatoxins, and may be suitable for complex mixtures as contour maps of spectra become available.

Monoclonal and polyclonal antibodies, which have been prepared against a variety of specific carcinogen-DNA adducts, can be used in specific and sensitive immunological techniques which have applications to human tissue samples. These competitive or noncompetitive immunoassays include the radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and ultrasensitive enzymatic radioimmunoassay (USERIA).

The ^{32}P -postlabeling techniques can successfully detect adducts of aromatic amines and amides, PAH, and methylating agents. Sensitivity of this assay is, in theory, the highest available to date for aromatic or bulky adducts.

^{32}P -Postlabeling Techniques

In 1981 Randerath, Reddy, and Gupta reported a ^{32}P -postlabeling method for the detection and quantitation of covalent adducts formed by the reaction of DNA with chemical carcinogens and mutagens. As depicted in figure 4, the

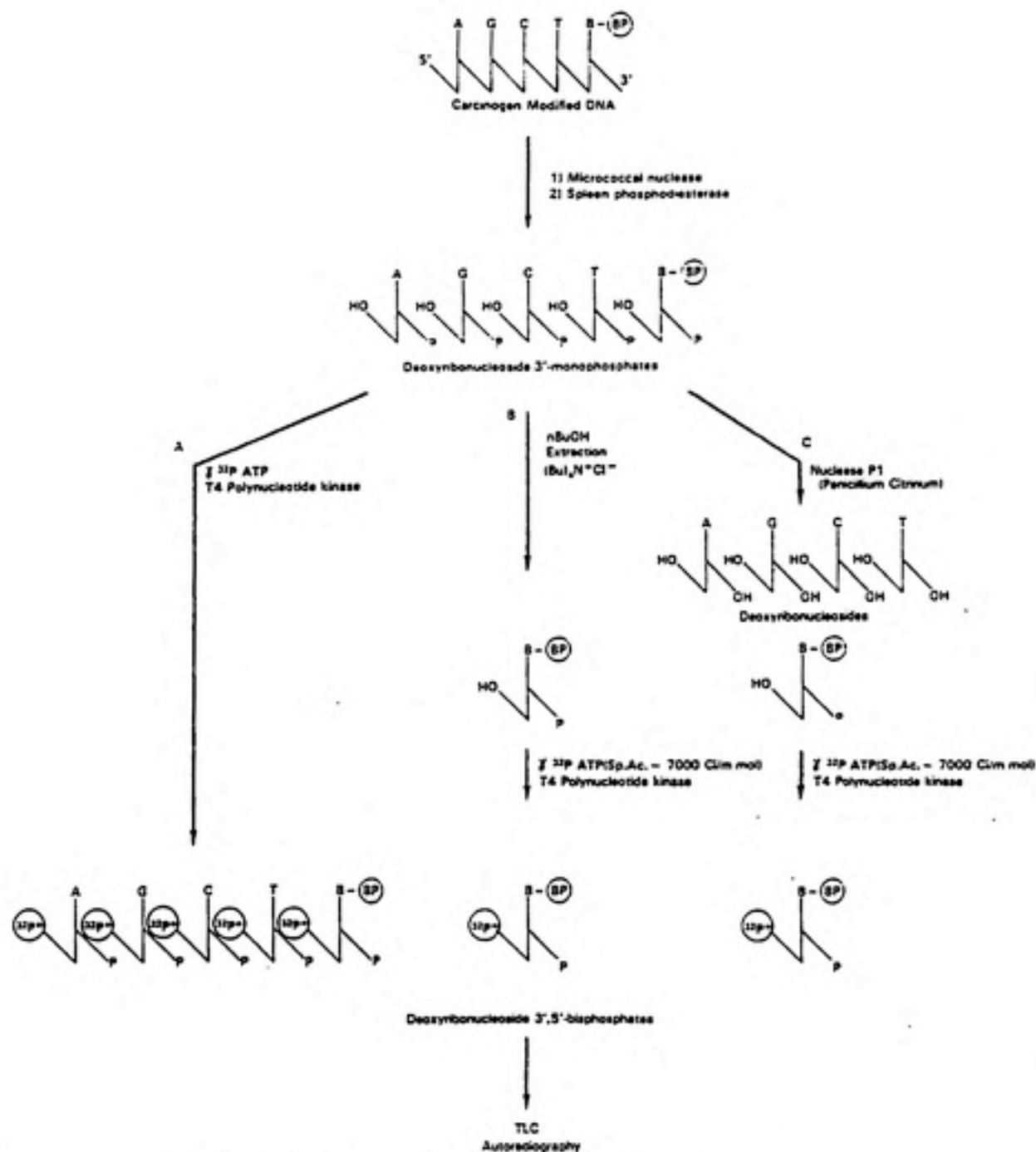


Fig. 4. ^{32}P -Postlabeling of DNA Adducts (from Watson 1987, p. 320). (A), Standard Method; (B), Butanol Extraction Enhancement; (C), Nuclease P1 Enhancement.

basic procedure involves enzymatic digestion of isolated DNA to deoxynucleoside 3'-monophosphates which are then converted to their corresponding 5'-³²P-labeled 3',5'-biphosphates by T4 polynucleotide kinase-catalyzed ³²P transfer from adenosine [γ -³²P]triphosphate. The mixture of labeled adducts is then purified and resolved by four-directional thin layer chromatography, usually anion exchange TLC on polyethyleneimine (PEI)-cellulose plates. Autoradiography is used to determine the presence of chemically modified nucleoside 3',5'-biphosphates. Adduct spots are then cut from the plates and measured using Cerenkov or liquid scintillation counting.

With the aim of increasing the sensitivity and accuracy of quantitative measurements, the original techniques were later modified with adduct enrichment procedures in a butanol extraction modification (Gupta 1985) and nuclease P1 modification (Reddy and Randerath 1986). The butanol adduct enrichment procedure, which involves extraction with 1-butanol in the presence of the phase-transfer agent tetrabutylammonium chloride prior to the labeling, is particularly suited to analysis of aromatic carcinogen-DNA adducts (especially PAH). It has been shown to enhance assay sensitivity to a level of 1 adduct per 10^9 - 10^{10} nucleotides with 1-10 μ g of DNA used (Gupta 1985; Gupta and Earley 1988).

Randerath et al. (1985) used the butanol extraction ³²P-postlabeling technique to determine binding levels of

individual PAH to mouse skin DNA in vivo, and they observed a good correlation between carcinogenic potency and binding. They failed to detect DNA binding of the noncarcinogens anthracene, pyrene, and perylene while the strong carcinogens B[a]P, 7,12-dimethylbenz[a]anthracene, and 3-methylcholanthrene exhibited highest levels of binding.

Watson (1987) and Randerath et al. (1985) review the significant advantages of the ^{32}P -postlabeling assay which include the following features: it is applicable to individual chemical compounds and mixtures; chemical identity of adducts doesn't have to be known to detect DNA binding; fingerprint patterns obtained after autoradiography of known chemicals can be used for identification of unknown chemicals and mixtures; it is highly sensitive for aromatics enabling detection of a few adducts per mammalian genome; it requires a minimal amount (1-10 ug) of DNA; it allows accurate quantitation of adducts; and it is potentially useful for studying repair and removal of adducts from cell or tissue DNA.

Application to DNA Adducts of Cyclopenta-PAH.

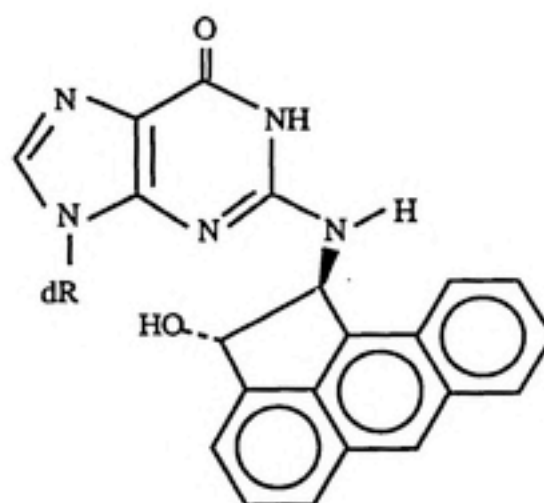
Recently Nesnow et al. (1989) analyzed and identified aceanthrylene-DNA adducts in C3H10T1/2 cells by using the butanol extraction modification of the ^{32}P -postlabeling technique. C3H10T1/2 cells treated with aceanthrylene for 24 hours formed four major adducts which were determined to be ACE-1,2-oxide-deoxyguanosine adducts (cyclopenta epoxide

adducts). Identities of this ACE metabolite and DNA base portion of the adducts were confirmed by co-chromatography of adduct mixtures from ACE-treated C3H10T1/2 cells, calf thymus DNA incubated with ACE-1,2-oxide, and the homopolymers of 2'-deoxyguanosine modified by ACE with Aroclor-1254-induced rat liver S9 activation.

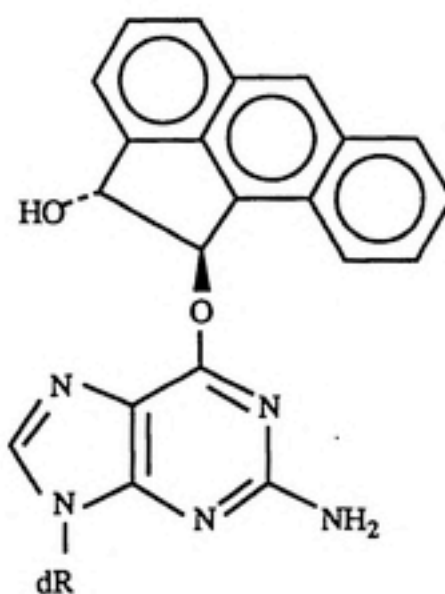
Because of the large delocalization energy ($\Delta E_{deloc}/\beta$) associated with the C-1 carbonium ion formed from the opening of ACE-1,2-oxide (Sangaiah et al. 1986), possible structures of the ACE-DNA adducts might be isomers of N²-[1-(trans-2-hydroxy-aceanthrylenyl)]-2'-deoxyguanosine and O⁶-[1-(trans-2-hydroxy-aceanthrylenyl)]-2'-deoxyguanosine, as shown in figure 5 (Nesnow et al. 1989).

In order to accomplish the detailed structural characterization of these adducts, co-chromatography with known chemical synthetic standards is needed. Results from this study support the choice of ACE-1,2-oxide-deoxyguanosine adducts as targets for synthetic standards, with exocyclic amino group modification given top priority based on high susceptibility of this nucleophilic position to covalent reaction with arene oxides.

Benz[*j*]aceanthrylene adducts in C3H10T1/2 cells have been studied in a similar manner using the same ³²P-postlabeling techniques (Lasley et al. 1990). DNA adduct autoradiograms were generated after treatment of the cells with B[*j*]A-1,2-oxide, B[*j*]A-9,10-dihydro-9,10-diol, the B[*j*]A bay region anti diol-epoxide, and B[*j*]A-1,2-dihydro-



A



B

Fig. 5. Possible Structures of Aceanthrylene-Deoxyguanosine Adducts. (modified from Nesnow et al. 1989, p. 232).

(A), N²-[1-(trans-2-hydroxyaceanthrylenyl)]-2'-deoxyguanosine;
 (B), O⁶-[1-(trans-2-hydroxyaceanthrylenyl)]-2'-deoxyguanosine.

1,2-diol. These autoradiograms were compared with those produced by treatment of homo polydeoxyguanylic acid with B[j]A-1,2-oxide and the B[j]A bay region anti diol-epoxide. Major adducts were determined to be formed by reaction of the bay region diol-epoxide with deoxyguanosine with minor contributions from reaction of B[j]A-1,2-oxide (the cyclopenta epoxide) with deoxyguanosine. Synthetic standards of deoxyguanosine modified at N-2 by the B[j]A diol-epoxide and B[j]A-1,2-oxide are needed in order to confirm the structures of these DNA adducts to a higher degree of specificity than base/metabolite composition.

This type of identification procedure, involving the use of chromatographic standards in the ^{32}P -postlabeling assay, has been reported in the literature. Pongracz et al. (1989) utilized chemical synthetic standards in the ^{32}P -postlabeling assay to identify two of the six DNA adducts formed by styrene oxide, which corresponded to aralkylation at O-6 of guanine.

Use of DNA Adducts in Human Biomonitoring

Traditionally, human exposure to occupational and environmental chemicals has been assessed by measuring concentrations of a substance in ambient environmental media - air, food, and water. However, biological monitoring, measurements that can be made on cells, tissues, or body fluids of exposed people, is presumably more directly relevant to the assessment of human health risks than any

environmental monitoring method (Miller 1984; Wogan and Gorelick 1985; Farmer, Neumann, and Henschler 1987; Wogan 1989).

Biological monitoring indicates the "internal dose" or "biologically effective dose" on an individual basis and takes into account variation in absorption, distribution, and metabolism of xenobiotics (Wogan and Gorelick 1985; Wogan 1989; Farmer, Neumann, and Henschler 1987).

Measurements of levels of the parent compound or its metabolite(s) in body tissues/fluids or excreta (usually blood, urine, and expired air) provides information on the "internal dose" of a chemical substance, which reflects environmental/occupational exposure (Miller 1984; Perera 1987). "Biologically effective dose" (also known as "target dose" or "molecular dose") refers to the amount of a chemical agent (mutagen/carcinogen) or its metabolite (ultimate carcinogen) that has reacted with critical cellular macromolecules (DNA, RNA, and/or proteins) of the target tissue or its surrogate (Perera 1987; Farmer, Neumann, and Henschler 1987).

For human biological monitoring (or biomonitoring), markers of biologically effective dose include the following: covalently bound DNA adducts, excreted DNA adducts, protein (i.e. hemoglobin or albumin) adducts, somatic cell mutations, micronuclei in lymphocytes, chromosomal aberrations, sister chromatid exchanges, unscheduled DNA synthesis, and oncogene activation

(Perera 1987). Figures 6(A) and 6(B) portray the role of DNA adduct determination as a biomonitoring technique in the overall assessment of human exposure to environmental mutagens/carcinogens.

Wogan and Gorelick (1985) and Harris (1985) discuss the application of carcinogen-DNA adducts as "internal dosimeters" of exposure to genotoxic chemicals. Qualitative and quantitative identification of DNA adducts in accessible human tissue (such as white blood cells or biopsy samples) can provide indication of an individual's exposure history as well as one's ability to metabolize a mutagen/carcinogen to its reactive form. As shown in figure 6(C), many factors affect the levels of carcinogen-DNA adducts detected in cells at any given time including the exposure-sampling interval, carcinogen exposure concentration, the metabolic balance between carcinogen activation and detoxication, and DNA repair rates (Harris 1985).

Although information from some experimental animal models suggests that levels of DNA adducts in tissues are linearly related to carcinogen dose (Wogan and Gorelick 1985), meaningful interpretation of DNA adduct levels as quantitative measures of exposure in humans is much more complex. This is due to the factors previously discussed as well as diverse human exposures to chemicals from a variety of sources beyond the workplace, all of which may influence adduct levels in a manner not easily determined. The ability of carcinogen-DNA adduct levels to accurately

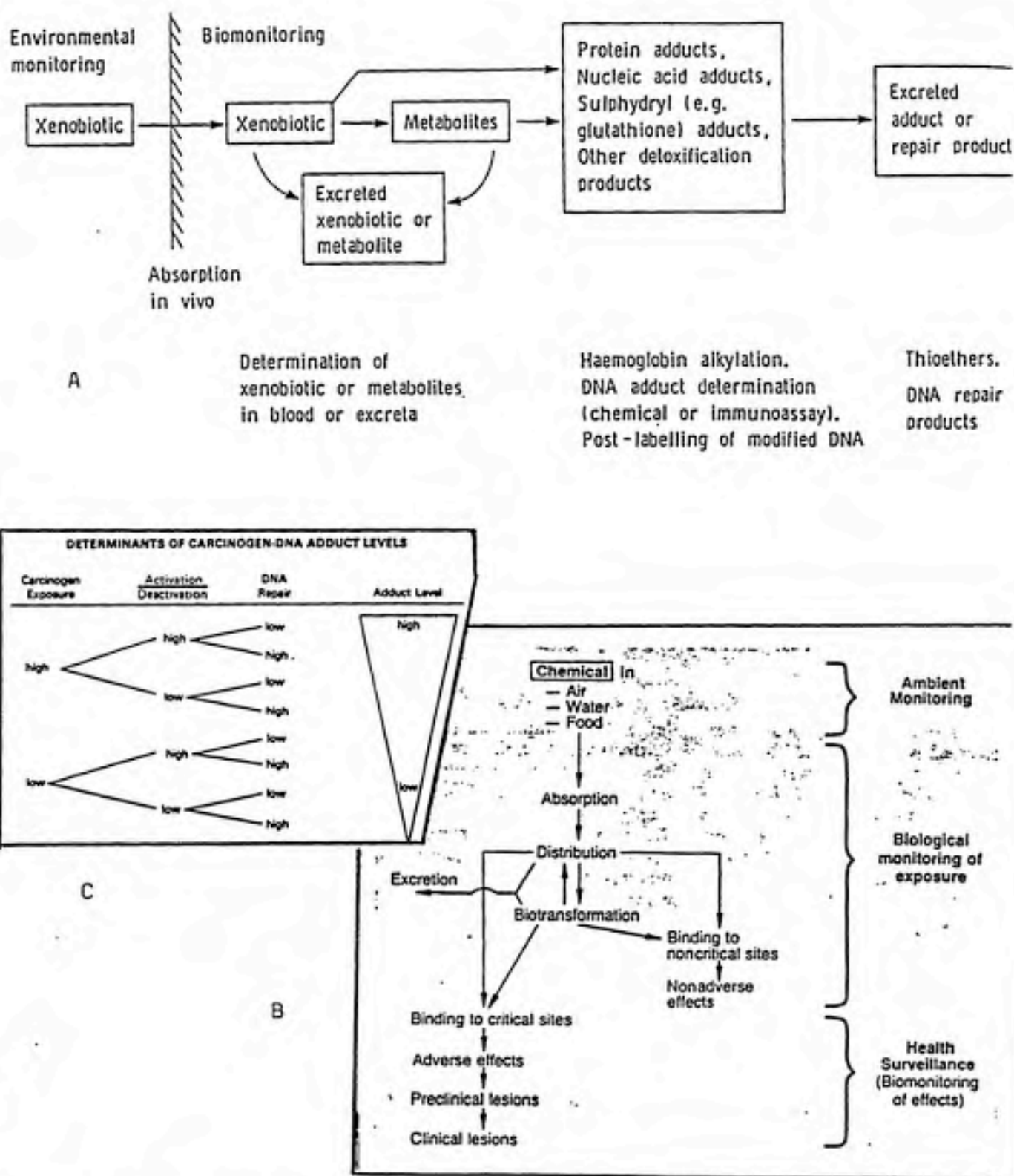


Fig. 6. Use of DNA Adducts in Human Biomonitoring. (A), Methods Used for Biomonitoring (from Farmer, Neumann, and Henschler 1987, p. 252); (B), Biomonitoring in Assessment of Human Exposure (from Miller 1984, p. 189A); (C), Determinants of Adduct Levels (from Harris 1985, p. 187).

reflect past or recent chemical exposures (adduct persistence) and acute or chronic exposures needs to be extensively investigated. According to Wogan and Gorelick (1985) and Harris (1985), DNA adducts can currently serve as useful qualitative markers of exposure.

Regarding the use of carcinogen-DNA adduct levels as indicators of long-term cancer risk, further complications arise including the validity of surrogate cell measurements in lieu of actual target tissue at risk and the necessary simplifying assumptions made about the multistage carcinogenic process (see Harris 1985; Perera 1987; Farmer, Neumann, and Henschler 1987 for information on DNA adducts in cancer risk assessment). Perera (1987) thoroughly reviews the growing field of molecular cancer epidemiology which involves conducting studies that establish relationships between biologic markers of dose and cancer risk in human populations.

Detection of Aromatic DNA Adducts in Human Tissues by ³²P-Postlabeling

Wogan (1989) thoroughly reviews the numerous human biomonitoring studies that have involved detection of DNA adducts of genotoxic agents in the cells and tissues of exposed persons. In the past, many have focused on detection of adducts arising from the bay region diol-epoxide (BPDE) metabolite of B[a]P through immunoassays, such as ELISA or USERIA, or physicochemical methods like SFS. BPDE adducts have been identified in DNA from white

blood cells of cigarette smokers, foundry workers, aluminum plant workers, and coke oven workers (see review by Wogan 1989).

Recently, numerous studies, many involving smokers, have employed the ^{32}P -postlabeling techniques for the detection of aromatic DNA adducts in human cells or tissues. Everson et al. (1986) investigated the presence of DNA adducts formed in placentas of women who smoked during pregnancy, and they found a major aromatic adduct strongly related to maternal smoking.

Through co-chromatography in the postlabeling assay, Randerath et al. (1986) compared DNA adducts detected in mouse skin treated with cigarette tar with DNA adducts detected in tissues from the bronchus and larynx of smokers. They observed several identical adducts present in the DNA samples from exposed mice and smokers.

DNA from normal human bone marrow cells was analyzed by ^{32}P -postlabeling, and ten out of ten individuals showed the presence of aromatic adducts that were not detected in human fetal bone marrow (Phillips, Hewer, and Grover 1986). Their findings suggest that adducts resulted from environmental exposure to unidentified genotoxic chemicals.

Chacko and Gupta (1987) analyzed DNA from human oral mucosal cells of smokers and nonsmokers. Two chromatographically distinct major adducts were detected in most smokers but not in nonsmokers. In addition, they report the use of known aromatic carcinogen-DNA adducts as

chromatographic standards to aid in the chemical identification of the mucosal DNA adducts. These co-chromatography experiments eliminated the possibility that any lesions were formed from reaction of B[a]P with N² of guanine or reaction of 4-aminobiphenyl with C-8 of guanine.

Phillips et al. (1987) detected aromatic adducts in DNA from white blood cells of foundry workers classified according to their exposure to airborne B[a]P in the workplace. Interestingly, none of the detected adducts in the individuals' DNA showed the chromatographic behavior expected of B[a]P-DNA adducts, and adduct spots were detected in a few unexposed workers. These results indicate that there may be significant interindividual variations in DNA adduct levels among similarly exposed workers.

DNA from white blood cells and placentas of non-smoking women exposed to residential wood combustion smoke during pregnancy was analyzed by the postlabeling assay for PAH-DNA adducts (Reddy, Kenny, and Randerath 1987). All placental DNA postlabeling maps (from exposed and nonexposed women) showed the presence of nine unidentified adducts that did not appear on WBC DNA maps. Their results suggest that residential wood combustion smoke did not produce DNA adducts at detectable levels and that placental DNA contained DNA adducts unrelated to wood smoke exposure.

CHAPTER III
EXPERIMENTAL MATERIALS AND METHODS

Basis for Synthetic Approach

Trans β -amino alcohol derivatives (enantiomeric mixtures) of acenaphthylene and aceanthrylene were previously synthesized and successfully used in condensation reactions with 6-chloropurine-9- β -D-ribofuranose to give N⁶-adenosine adducts, RNA adducts expected from the corresponding 1,2-oxides (Bartczak et al. 1989). The same CP-PAH derivatives were later successfully condensed with 6-chloropurine-9-(2-deoxy- β -D-ribose) to produce N⁶-deoxyadenosine adducts (Gold: unpublished results).

For synthesis of N²-modified deoxyguanosine (DNA adducts), the appropriate halopurine precursor is a 2-halo-2'-deoxyinosine. Generation of the 2-chloro derivative had been attempted previously in this laboratory and was achieved by Lewis acid catalyzed glycosylation of 2-chlorohypoxanthine (from partial hydrolysis of 2,6-dichloropurine) with 2-deoxy-3,5-di-O-p-toluoyl- α -D-erythro-pentofuranosyl chloride (unpublished results). However, due to high cost of 2,6-dichloropurine and presumed increased efficiency of the condensation reaction expected by the more reactive brominosine derivative, synthesis of 2-bromo-2'-deoxyinosine was investigated.

Recently, Hildebrand et al. (1990) indicated use of 2-bromo-2'-deoxyinosine (2-BrdI) in generation of N²-n-hexyl-2'-deoxyguanosine, but no details of the synthesis of 2-BrdI were provided in the literature. In addition, although N²-aryl and N²-alkyl 2'-deoxyguanosine derivatives have been reported in the literature (Wright and Dudycz 1984; Casale and McLaughlin 1990; Lee et al. 1990; Hildebrand et al. 1990), no synthesis of N²-modified-2'-deoxyguanosine involving a cyclopenta-PAH has been published.

Efficient synthesis of 2-bromohypoxanthine (1) has been reported in the literature (Beaman, Gerster, and Robins 1962). The Lewis acid catalyzed glycosylation of 2-bromohypoxanthine with the anomeric mixture 1,3,5-tri-O-acetyl-2-deoxy- α,β -D-erythro-pentofuranose (synthesized according to Gold and Sangaiah 1990) yielded a mixture of N⁷ α plus β and N⁹ α plus β isomers which couldn't be readily separated (Gold: unpublished results).

To avoid problems previously encountered in separating isomeric glycosylation products and to presumably enhance yields of N⁹ isomers over N⁷ isomers, synthesis of O⁶-NPE (p-nitrophenylethyl group)-protected 2-bromohypoxanthine (4) by the method of Raju, Robins, and Vaghefi (1989) was undertaken in this study, as shown in reaction scheme I of figure 7. The synthetic strategy was originally based on Lewis acid catalyzed glycosylation of the NPE-protected 2-bromohypoxanthine (4) with anomeric 1,3,5-tri-O-acetyl-2-

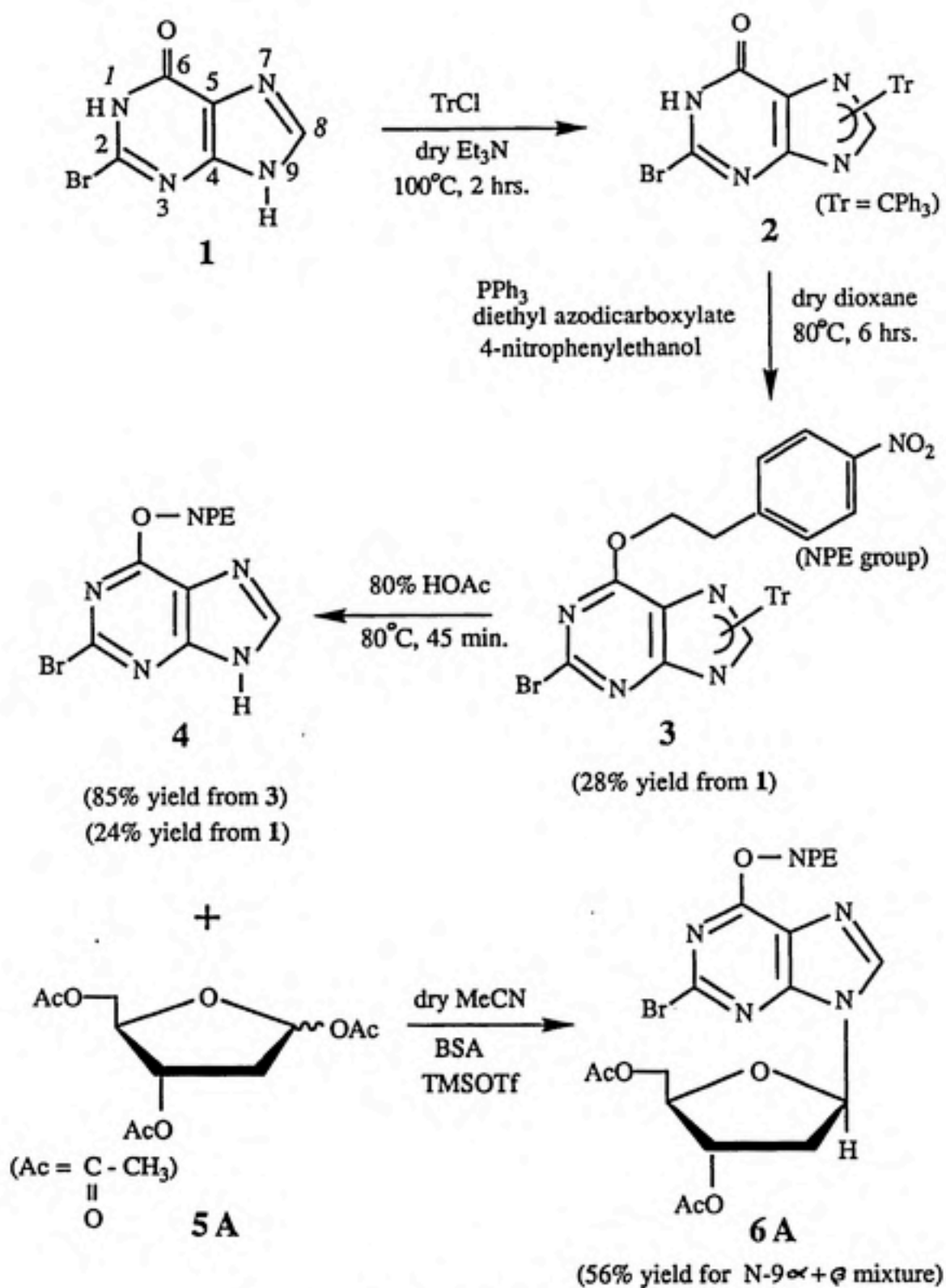


Fig. 7. Reaction Scheme I

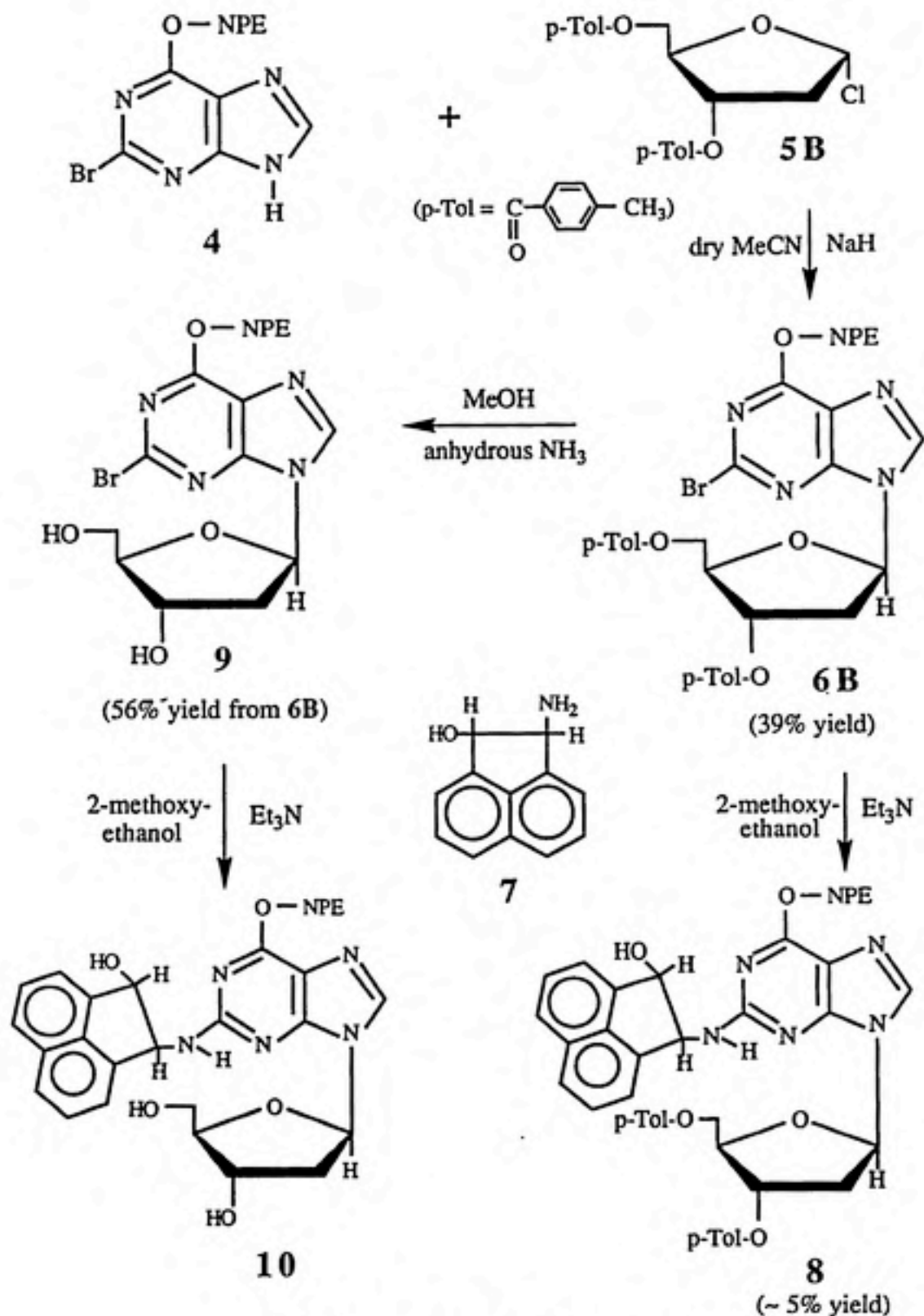


Fig. 8. Reaction Scheme II

deoxy- α,β -D-erythro-pentofuranose (5A), as shown in figure 7. However, the synthetic scheme was later modified due to difficulties in efficiently separating the N⁹ α and β isomeric glycosylation products (6A).

Thus, reaction scheme II (shown in figure 8) was developed. This involved a sodium hydride catalyzed glycosylation of O⁶-NPE-protected 2-bromohypoxanthine (4) with 2-deoxy-3,5-di-O-p-toluoyl- α -D-erythro-pentofuranosyl chloride (5B). The direct glycosylation product (6B) was then coupled with trans acenaphthene 1-amino-2-ol (7) to generate protected N²-deoxyguanosine adducts (8).

In hopes of improving yields of the final adduct products, ammonolysis reactions were carried out on the glycosylation product (6B) to remove the bulky p-toluoyl groups (giving O⁶-NPE-protected 2-BrdI, 9) prior to condensation with the cyclopenta-PAH derivative. Condensation reactions were conducted with the NPE group still intact on the O-6 position of the purine because the resulting aromatic 6-membered ring is expected to enhance the nucleophilic substitution reaction at C-2.

Instrumentation and Laboratory Materials

¹H nuclear magnetic resonance (NMR) spectra were acquired at the UNC Department of Chemistry on a Varian XL-400 at 400 MHz. Chemical shifts are given in parts per million (ppm) relative to tetramethylsilane (TMS). Mass spectra were obtained at the UNC Department of Environmental

Sciences and Engineering on a VG 70S 250SEQ mass spectrometer using a fast atom bombardment (FAB) source. UV-visible spectra were recorded on a Spectronic 1201 spectrophotometer (Milton Roy Company).

High performance liquid chromatography (HPLC) was performed with an ISCO Model 2360 gradient programmer and 2300 pump connected to a DuPont Zorbax C-8 9.4 X 250 mm column. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Preparative thin layer chromatography (TLC) was conducted using Silica Gel PK5F (20 X 20 cm) 500 um/1000 um plates (Whatman) or Reversed Phase PLKC18F Linear-K (20 X 20 cm) 1000 um plates (Whatman). Analytical TLC was performed using Silica Gel on Aluminum (5 X 10 cm) plates (Aldrich). Column chromatography was conducted using Merck Silica Gel, grade 60, 230-400 mesh, 60 Å obtained from Aldrich Chemical Company, Inc.

All solvents were reagent grade, and they were dried over CaH/P₂O₅ and distilled prior to use where indicated. All routine chemicals were purchased from one of the following commercial sources: American Scientific Products (Charlotte N.C. distribution center); Aldrich Chemical Company, Inc. (Milwaukee, Wis.); Sigma Chemical Company (St. Louis, Mo.); and Fisher Scientific (Fair Lawn, N.J.).

Synthesis of O⁶-NPE-Protected 2-Bromohypoxanthine (4)

2-Bromohypoxanthine was converted to its O⁶-NPE-protected derivative based on the three-step procedure of Raju, Robins, and Vaghefi (1989) with slight modifications regarding work-up. The reaction sequence, shown in figure 7, was repeated twice in order to generate sufficient amounts of 2-bromo-O⁶-p-nitrophenylethylpurine for use in future glycosylation reactions. Typical reaction procedures were as follows.

2-Bromohypoxanthine (1)

The starting material (1) was prepared by Dr. Sangaiah according to the procedures of Beaman (1954) and Beaman, Gerster, and Robins (1962). The reaction sequence entailed purine ring closure of 2-mercapto-4,5-diamino-6-hydroxypyrimidine to produce 2-mercapto-6-hydroxypurine followed by replacement of the mercapto group with bromine to give the bromopurine (1). UV-visible spectrum (ethanol) of 1 agreed with reported values (Beaman 1954): $\lambda_{\text{max}} = 252.2$ nm, and the structure was confirmed by ¹H NMR and FAB MS.

Tritylated 2-Bromohypoxanthine (2)

A mixture of compound 1 (0.5 g, 2.33 mmol), trityl (Tr) chloride (1.3 g, 4.66 mmol), and dried, distilled triethylamine (3 mL, 21.5 mmol) was heated at 100°C with stirring for 2 h. Triethylamine was removed on a rotary evaporator under oil pump vacuum at 65°C, and the entire

sludgy yellow-brown residue containing 2 was carried on to the next step.

Tritylated 2-Bromo-O⁶-p-nitrophenylethylpurine (3)

To the crude compound 2 from the previous step, was added 4-nitrophenylethanol (0.78 g, 4.66 mmol), triphenylphosphine (1.22 g, 4.66 mmol), and diethyl azodicarboxylate (0.8 mL, 5.1 mmol) in dried, distilled 1,4-dioxane (15 mL). The mixture was heated at 80°C with stirring for 6 h. To quench the reaction, deionized water (15 mL) was added with stirring for 5 min. Dioxane and water were removed on a rotary evaporator under oil pump vacuum at 70°C to give a syrupy brown-yellow residue.

Attempted crystallization of 3 from this residue in hot 2-propanol gave a yellow crystalline precipitate, which contained phenyl-substituted impurities according to ¹H NMR analysis (probably trityl alcohol). This precipitated product was purified by column chromatography on silica gel with benzene:chloroform (1:3) as eluant, gradually increased to 100% chloroform. The N-9 isomer of desired product 3 eluted before the N-7 isomer.

Appropriate fractions were combined and solvents removed on a rotary evaporator and oil pump to afford a white crystalline solid for the N-9 isomer (280 mg) and a light yellow crystalline solid for the N-7 isomer (115 mg) of pure compound 3. Yield of 3 from 1 was 28%. ¹H NMR

spectrum (400 MHz, CDCl_3) of 3 concurred with reported values from Raju, Robins, and Vaghefi (1989) (see figure 9).

2-Bromo- O^6 -p-nitrophenylethylpurine (4)

To pure compound 3 from the previous step was added an 80% acetic acid solution (20 mL), and the suspension was heated at 80°C with stirring. The reaction was monitored by analytical TLC and stopped at 45 min after 3 had dissolved in the solution. Acetic acid and water were removed on a rotary evaporator under oil pump vacuum at 70°C, and an off-white crystalline solid was the product. Trituration (2 X) of this reaction product with chloroform gave a precipitate, and the mixture was filtered under vacuum to obtain an off-white crystalline solid and a light yellow filtrate solution.

Analytical TLC and ^1H NMR analysis (400 MHz, DMSO-d_6) showed that the off-white crystalline precipitate was a single product, identified as compound 4 (200 mg) by the ^1H NMR spectrum which agreed with published data (Raju, Robins, and Vaghefi 1989) (see figure 10). Yield of 4 from 3 was 85%, and the overall yield of 4 from 1 was 24%. Compound 4 was evacuated on an oil pump at 75°C for 10 h to ensure complete removal of traces of water prior to proceeding with a glycosylation reaction.

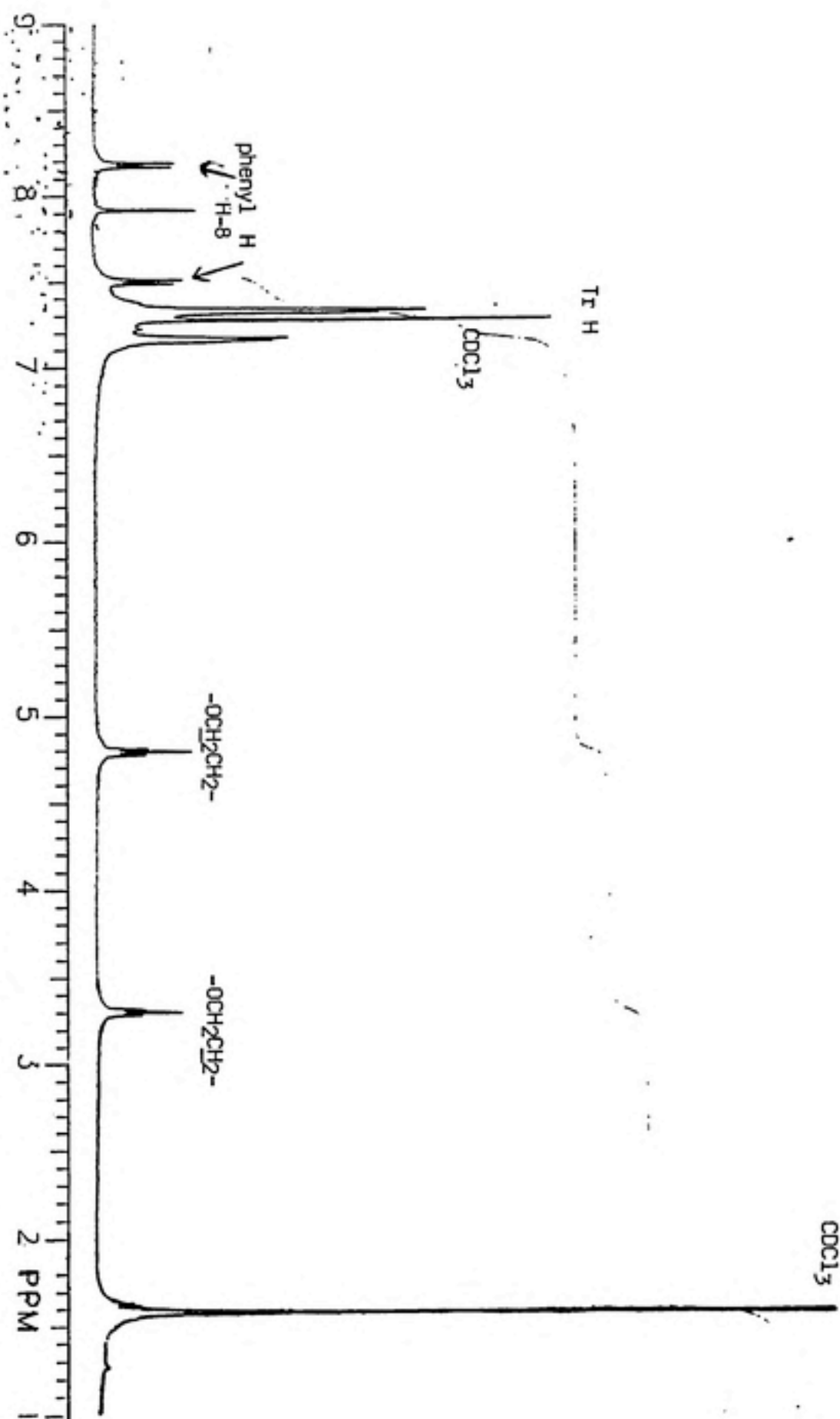
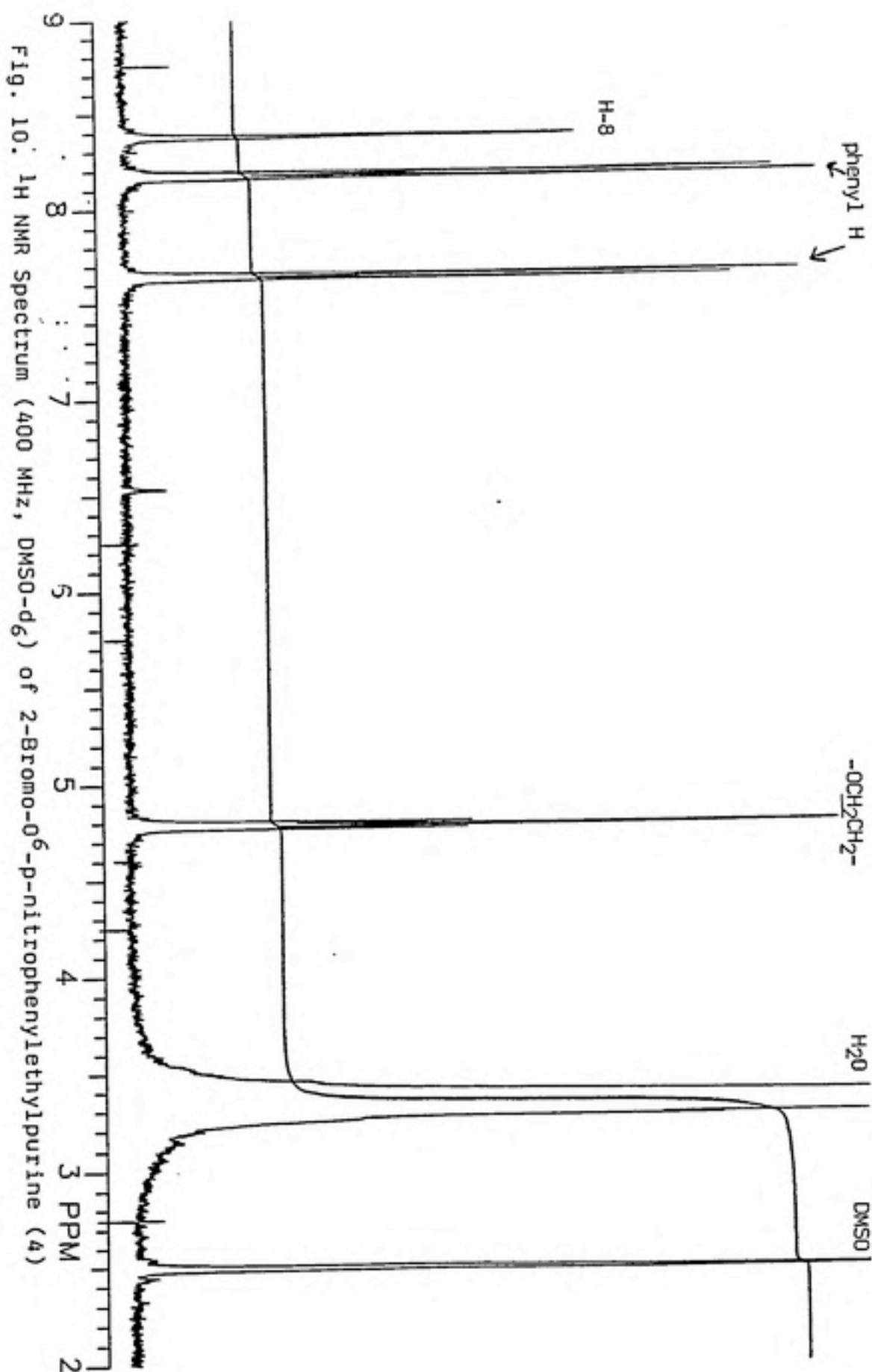


Fig. 9. ^1H NMR Spectrum (400 MHz, CDCl_3) of tritylated 2-bromo-06-p-nitrophenylethylpurine (3) N-9 isomer.



Lewis Acid Catalyzed Glycosylation of O⁶-NPE-Protected
2-Bromohypoxanthine (4)

The Lewis acid catalyzed glycosylation reaction was carried out according to the conditions of Raju, Robins, and Vaghefi (1989) with work-up methods taken from Wright and Dudycz (1984). The reaction sequence, shown in figure 7, involved the following procedures.

1,3,5-Tri-O-acetyl-2-deoxy- α,β -D-erythro-pentofuranose (5A)

The deoxyribose derivative 5A was prepared by Dr. Gold using a three-step published procedure (Gold and Sangaiah 1990) beginning with 2-deoxy-D-erythro-pentofuranose. An ¹H NMR spectrum (400 MHz, CDCl₃) of 5A was taken prior to use in the glycosylation reaction, and the spectrum confirmed its structure and purity in agreement with the referenced values.

2-Bromo-O⁶-p-nitrophenylethyl-9-(2-deoxy-3,5-di-O-acetyl- β -D-erythro-pentofuranosyl)purine (6A) and Its Isomers

Under an argon atmosphere, a white suspension of dried compound 4 (0.34 g, .95 mmol) in dried, distilled acetonitrile (10 mL) was treated with bistrimethylsilylacetamide (BSA) (0.7 mL, 2.84 mmol) and stirred for 1 h at room temperature, giving a clear yellow solution. To this solution was added compound 5A (0.25 g, .95 mmol) in dried, distilled acetonitrile (5 mL) with stirring followed by rapid addition of the catalyst TMSOTf (CF₃SO₃SiMe₃) (0.3 mL, 1.42 mmol). After stirring for 15 min, this solution was refluxed with stirring under argon for 1 h. The clear

brown solution was then stirred at room temperature overnight (16 h).

The acetonitrile was then evaporated at room temperature on a rotary evaporator under oil pump vacuum with a dry ice condenser, and the residual yellow syrup was dissolved in chloroform (50 mL). The solution was washed with water until the pH of the aqueous phase was neutral (4 X 25 mL) and then dried over anhydrous sodium sulfate. The chloroform was removed on a rotary evaporator at 55°C to give a light yellow solid product, which was purified by preparative TLC on silica gel developed with 1% methanol in chloroform.

The bands containing a mixture of N-9 α and β anomers of 6A ($R_f=0.4$) gave an off-white crystalline solid (300 mg) that was subjected to further prep TLC in an attempt to separate the anomers by using various combinations of eluants (2% isopropanol in chloroform proving best). However, complete resolution of the N-9 α and β anomeric mixture could not be achieved. The combined yield of N-9 α and β anomeric mixture of 6A was 56% with a ratio of β anomer/ α anomer of 45% /55%. The ^1H NMR (400 MHz, CDCl_3) spectrum (see figure 11) was in accord with expectations for the mixture of anomers. The salient features were a pseudo-triplet signal for H-1' of the β anomer and a doublet of doublets signal for H-1' of the α anomer.

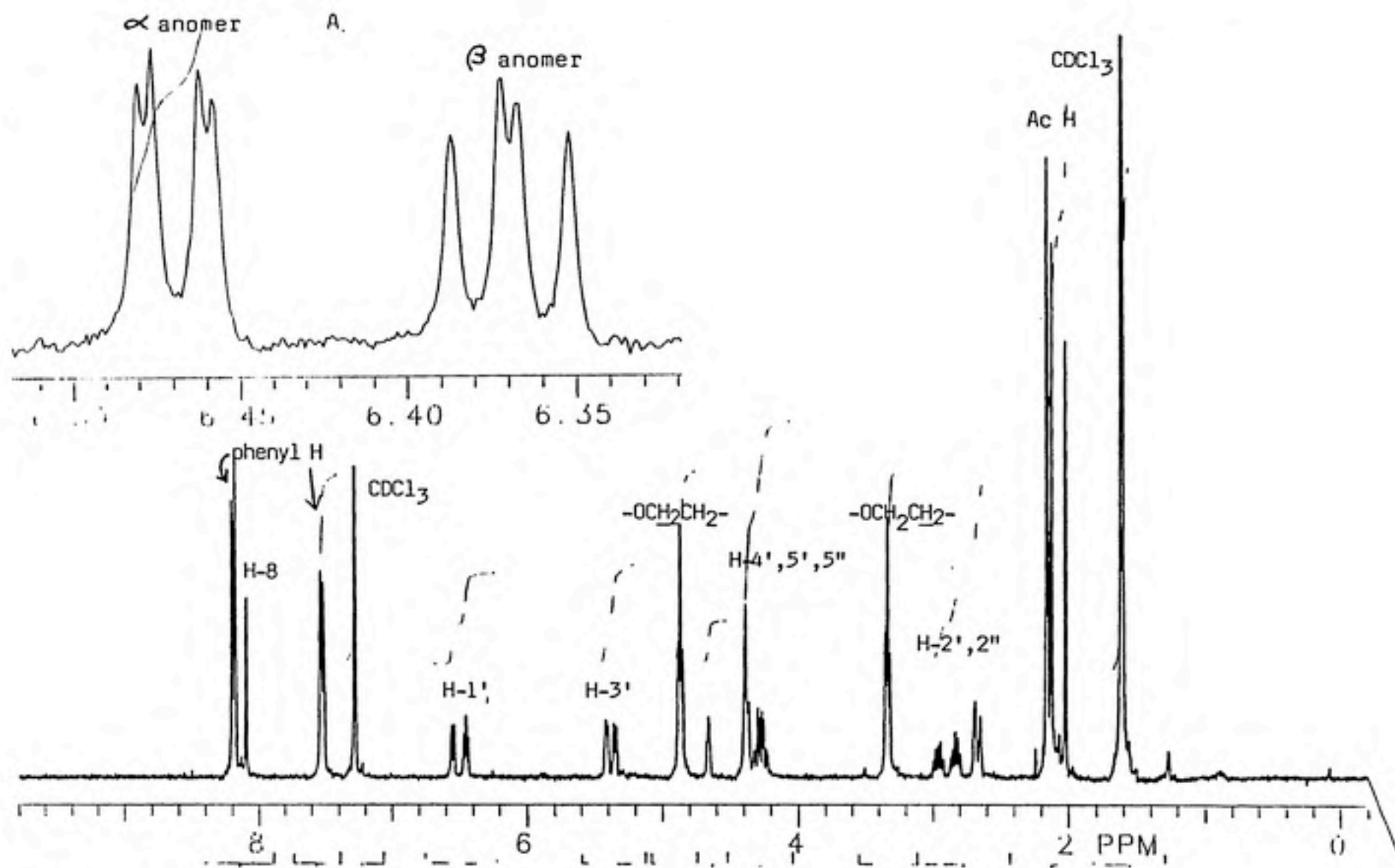


Fig. 11. ^1H NMR Spectrum (400 MHz, CDCl_3) of N-9 α and β anomeric mixture of 2-Bromo-06-p-nitrophenylethyl-9-(2-deoxy-3,5-di-O-acetyl- β -D-erythro-pentofuranosyl)purine (6A). (A), ^1H NMR signals for N-9 α anomer and N-9 β anomer, H-1'

Sodium Salt Glycosylation of O⁶-NPE-Protected
2-Bromohypoxanthine (4)

The sodium salt (sodium hydride catalyzed) glycosylation of 4 with chlorofuranose 5B was accomplished by the method of Kazimierczuk et al. (1984), Wright et al. (1987), and Kazimierczuk et al. (1990). The reaction sequence, shown in figure 8, was repeated twice in order to generate a sufficient amount of O⁶,3',5'-protected derivative of 2-bromo-2'-deoxyinosine (6B) for use in future condensation reactions. Typical reaction procedures were as follows.

2-Deoxy-3,5-di-O-p-toluoyl- α -D-erythro-pentofuranosyl chloride (5B)

The chlorofuranose 5B was synthesized exactly in accordance with the detailed procedures of Zorbach and Tipson (1968, pp. 521-22) so specifics will not be provided here. The three-step sequence involved conversion of 2-deoxy-D-erythro-pentose to methyl 2-deoxyribose followed by addition of p-toluoyl groups to 3 and 5 positions and then the final substitution of chlorine at the 1 (α) position of the sugar.

9.99 g (70% yield) of 5B was obtained, and its ¹H NMR spectrum (400 MHz, CDCl₃) verified its structure and purity (see figure 12).

2-Bromo-O⁶-p-nitrophenylethyl-9-(2-deoxy-3,5-di-O-p-toluoyl- β -D-erythro-pentofuranosyl)purine (6B) and Its 7- β Isomer

Under an argon atmosphere, sodium hydride powder (97%) was added to a stirred suspension of dried compound 4

FREQUENCY 399 862 MHz
 SPECTRAL WIDTH 5299 HZ
 ACQ TIME 2.852 SEC
 PULSE WIDTH 30 DEGREES
 AMBIENT TEMPERATURE
 NO. REPETITIONS 24
 SCAN RATE 20 HZ
 DOUBLE PRECISION ACQUISITION
 DATA PROCESSING
 LINE BROADENING 0.1 HZ
 FT SIZE 32K
 FID TIME 1.1 MINUTE

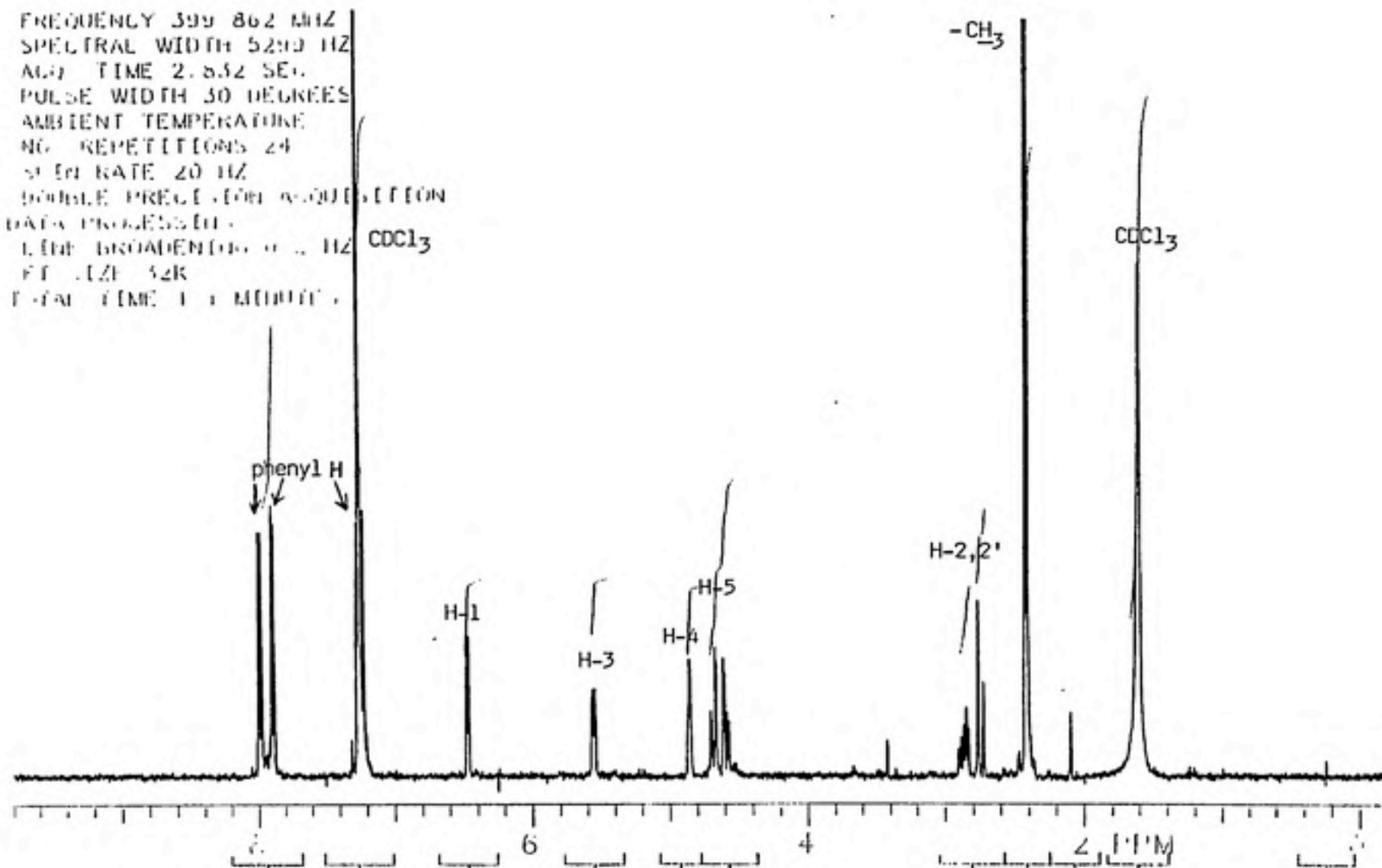


Fig. 12. ^1H NMR Spectrum (400 MHz, CDCl_3) of 2-Deoxy-3,5-di-O-p-toluoyl- α -D-erythro-pentofuranosyl chloride (5B)

(0.48 g, 1.32 mmol) in dried, distilled acetonitrile (20 mL), and the mixture was stirred for 30 min. Next, compound 5B was added in small portions over a 20 min period, and the cloudy mixture was stoppered and stirred overnight (16 h) at room temperature under an argon atmosphere.

The reaction mixture was filtered under vacuum using a sintered glass fine frit to remove a tan crystalline solid precipitate (95 mg). The acetonitrile was removed from the clear light yellow filtrate on a rotary evaporator at 80°C followed by further evacuation under oil pump vacuum for complete drying. The resulting light yellow solid product was purified by column chromatography on silica gel with 4% acetone in toluene as the initial eluant. After the N-9 B glycosylation isomer 6B eluted, 6% acetone in toluene was used to elute the N-7 B isomer of 6B that followed.

Appropriate fractions were combined and solvents evaporated on a rotary evaporator and oil pump to afford a pure off-white crystalline solid for the N-9 B isomer (368 mg, 39% yield) and a pure off-white crystalline solid (508 mg) for the N-7 B isomer.

For the N-9 B glycosylation isomer 6B: Melting point 82-84°C. ^1H NMR (400 MHz, CDCl_3) (see figure 13) - δ 2.41, 2.45 (3H each, 2s, CH_3); 2.88-2.93 (2H, m, H_2' , H_2''); 3.31 (2H, t, $J_{\text{ave}}=6.64$ Hz, OCH_2CH_2); 4.64-4.69, 4.74-4.77 (3H, m, H_4' , H_5' , H_5''); 4.84 (2H, t, $J_{\text{ave}}=6.47$ Hz, OCH_2CH_2); 5.77-5.78 (1H, m, H_3'); 6.56 (1H, pseudo t, $J_{\text{ave}}=6.11$ Hz, H_1'); 7.24, 7.29, 7.50, 7.89, 7.98, 8.18 (2H each, 6d, phenyl Hs); 8.07

FREQUENCY 399.562 MHz
 SPECTRAL WIDTH 5100 Hz
 ACQ. TIME 2.651 SEC.
 PULSE WIDTH 30 DEGREES
 AMBIENT TEMPERATURE
 NO. REPETITIONS 34
 SCAN RATE 20 HZ
 DOUBLE PRECISION ACQUISITION
 DATA PROCESSING
 LINE BROADENING 1 Hz
 FT SIZE 52K
 TOTAL TIME 1.1 MINUTE

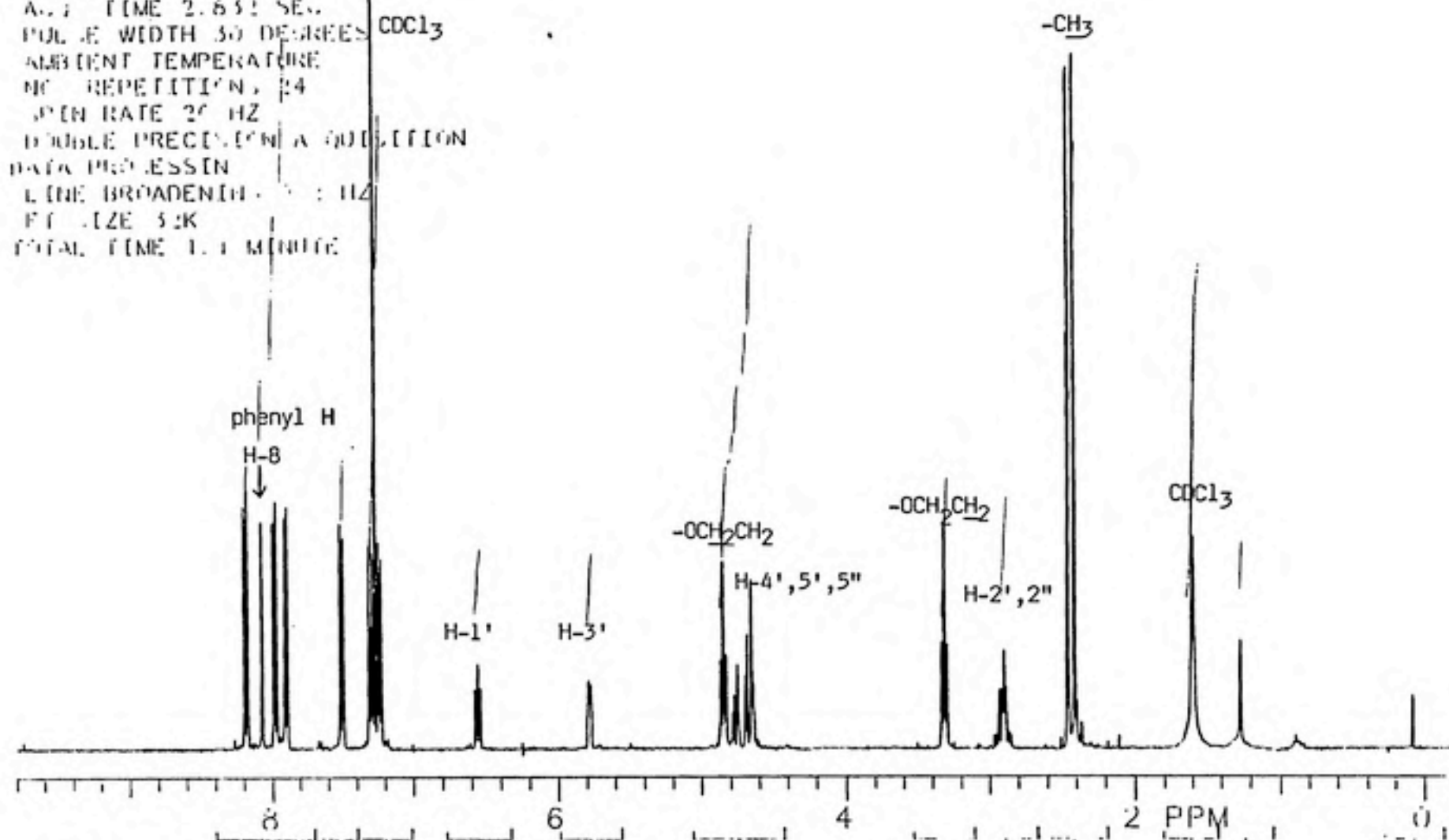


Fig. 13. ^1H NMR Spectrum (400 MHz, CDCl_3) of 2-Bromo- 0^6 -p-nitrophenylethyl-9-(2-deoxy-3,5-di-O-p-toluoyl- β -D-erythro-pentofuranosyl)purine (6B)

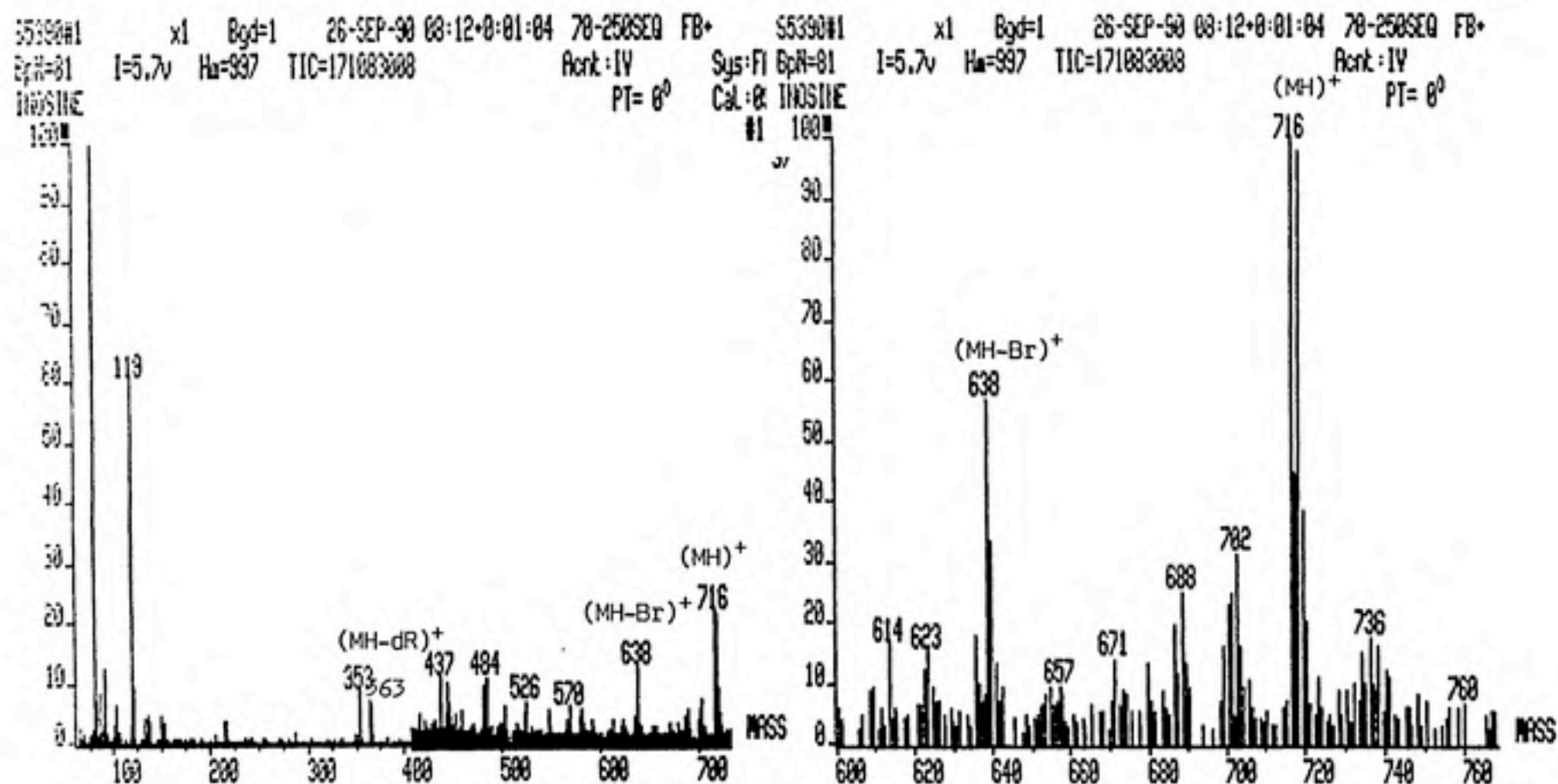


Fig. 14. FAB Mass Spectrum of 2-Bromo-O⁶-p-nitrophenylethyl-9-(2-deoxy-3,5-di-O-p-toluoyl-β-D-erythro-pentofuranosyl)purine (6B)

FREQUENCY 399.56 MHz
 SPECTRAL WIDTH 52.00 kHz
 ACQ. TIME 2.632 SEC.
 PULSE WIDTH 30 DEGREES
 AMBIENT TEMPERATURE
 NO. REPETITIONS 4
 SPIN RATE 20 Hz
 DOUBLE PRECISION ACQUISITION
 DATA PROCESSING
 LINE BROADENING 0.2 Hz
 F2 FREQ 52K
 TOTAL TIME 1.1 MIN.

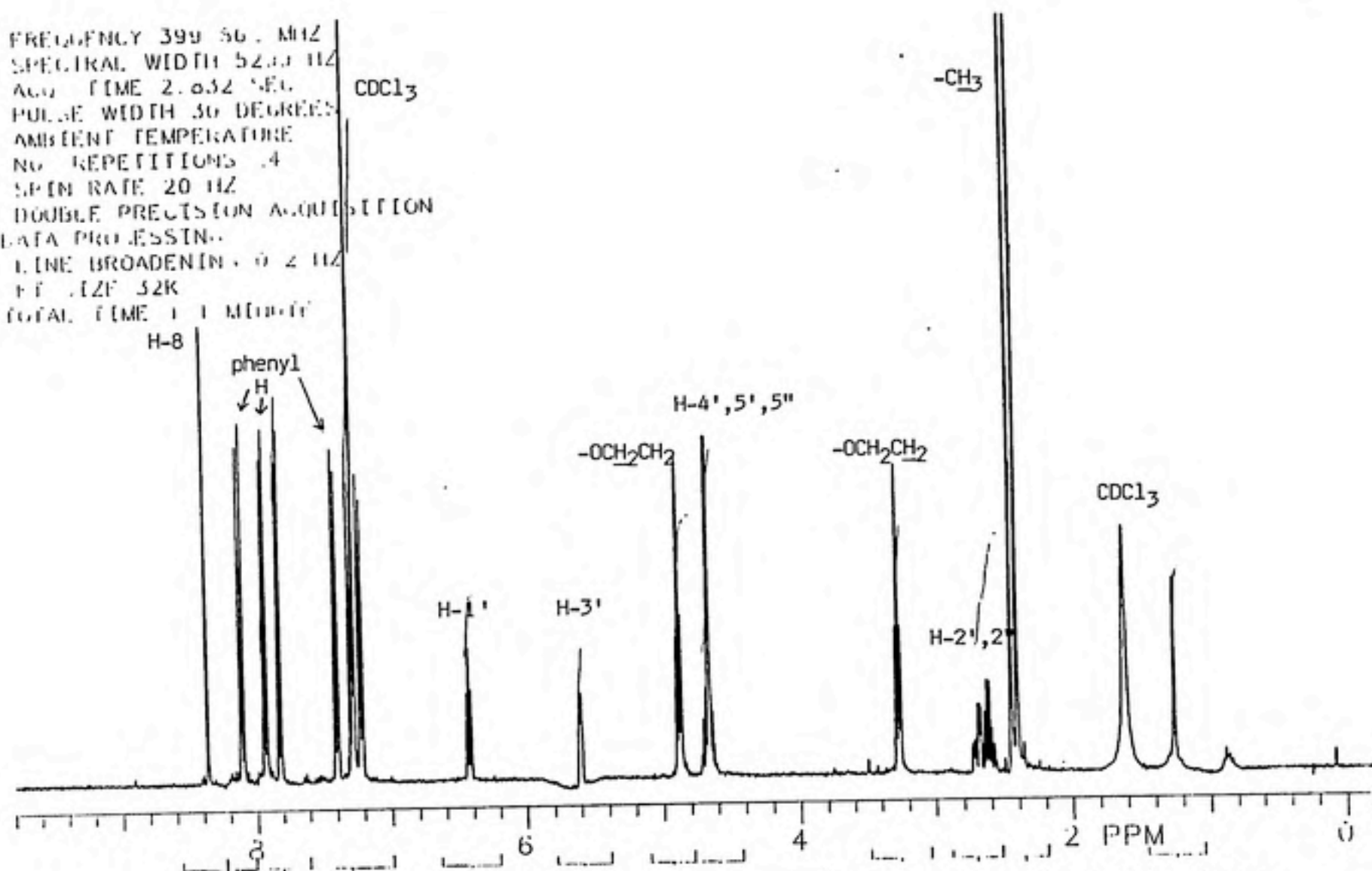


Fig. 15. ^1H NMR Spectrum (400 MHz, CDCl_3) of 2-Bromo- O^6 -p-nitrophenylethyl-7-(2-deoxy-3,5-di-O-p-toluoyl- β -D-erythro-pentofuranosyl)purine

(1H, s, H8). FAB Mass Spectrum (see figure 14) - m/z 716,718 (MH)⁺; 638 (MH-Br)⁺; 363,365 (MH-dR)⁺; Accurate Mass of MH⁺ - observed 716.1361, 0.9 ppm difference from calculated value for C₃₄H₃₀O₈N₅⁷⁹Br₁+H 716.1354.

See figure 15 for the ¹H NMR spectrum (400 MHz, CDCl₃) of the N-7 β glycosylation isomer of 6B.

Condensation of Glycosylation Product (6B)
with Cyclopenta-PAH amino alcohol (7)

The condensation reaction of 6B with 7 to synthesize a protected N²-deoxyguanosine adduct of a cyclopenta-PAH was based in part on the procedures of Bartczak et al. (1989), Hildebrand et al. (1990), and Wright and Dudycz (1984). The reaction sequence, shown in figure 8, included the following steps.

Trans acenaphthene 1-amino-2-ol (7)

The enantiomeric 1-amino-2-hydroxy cyclopenta-PAH mixture 7 was previously synthesized by Bartczak and co-workers according to their published procedures (1989).

N²-[1-(trans-2-hydroxy-acenaphthenyl)]-O⁶-p-nitrophenylethyl-9-(2-deoxy-3,5-di-O-p-toluoyl-β-D-erythro-pentofuranosyl) purine (8)

A mixture of 6B (58 mg, .081 mmol), cyclopenta-PAH amino alcohol derivative 7 (15 mg, .081 mmol), and triethylamine (.01 mL, .081 mmol) in 2-methoxyethanol (5 mL, b.p. 124°C) was refluxed with stirring for 24 h.

The solvent was removed on a rotary evaporator at 90°C to give a brown syrup which was placed on silica gel

preparative TLC plates developed with 5% methanol-19% acetone-76% toluene.

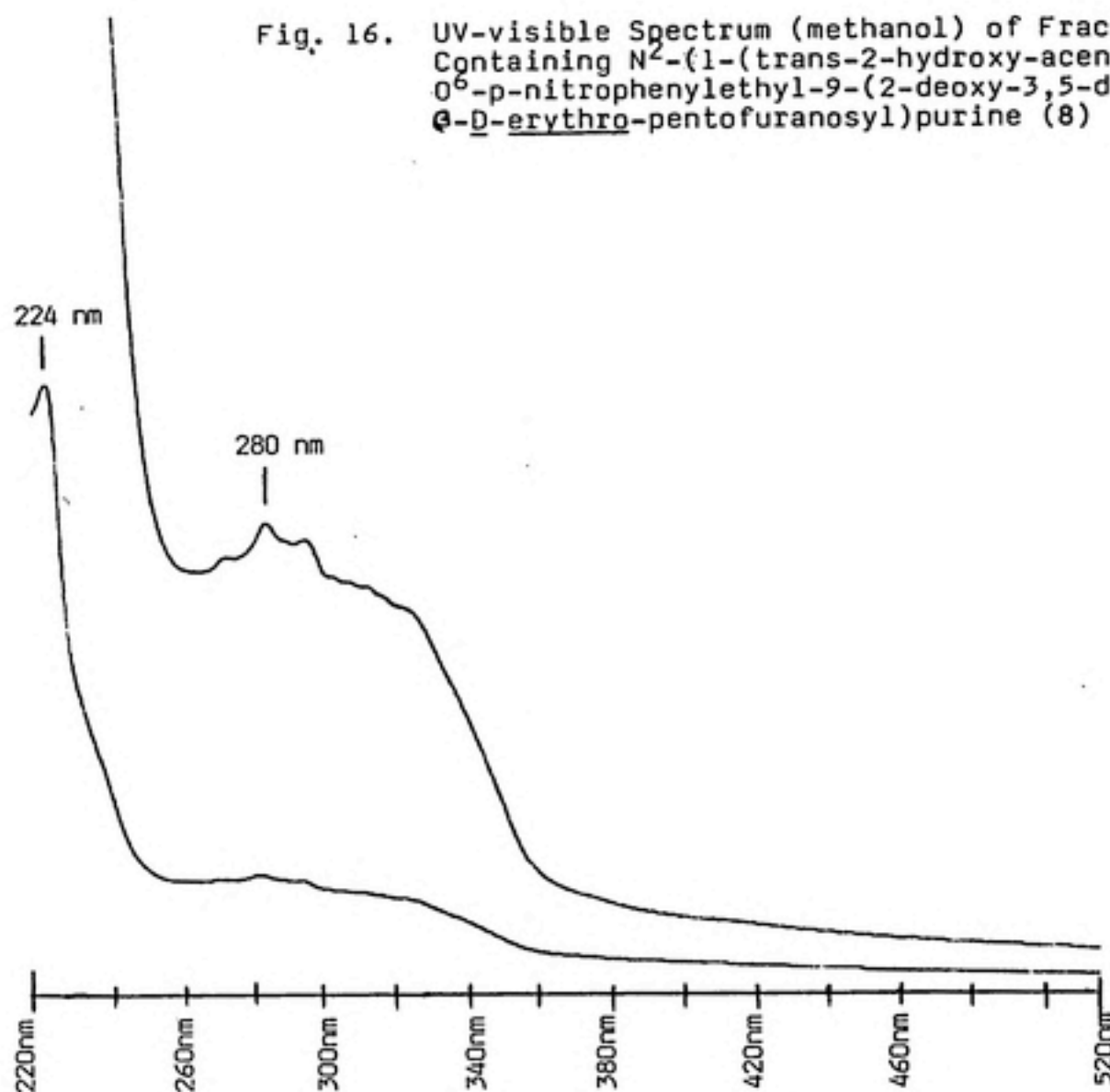
Products from ten resolved bands were screened by UV-visible spectroscopy for the acenaphthene chromophore, and the presence of N²-modified deoxyguanosine derivatives was confirmed by FAB mass spectrometry.

The fraction containing compound 8 ($R_f=0.70$) chromatographed directly below starting material 6B, fluoresced light blue under long wave UV, and was isolated as a light yellow oily residue (3.5 mg, 5%) which showed the following characteristics: UV-visible spectrum (methanol) (see figure 16) - $\lambda_{max} = 224,280 \text{ nm}$; FAB Mass Spectrum full scan (see figure 17(A)) - $m/z \text{ } 821 \text{ (MH)}^+$. FAB MS/MS (see figure 17(B)) - $m/z \text{ } 821.1315 \text{ (MH)}^+$; $803.1281 \text{ (MH-H}_2\text{O)}^+$; $469.1427 \text{ (MH-dR+H)}^+$; $451.6453 \text{ (MH-dR-H}_2\text{O+H)}^+$.

Ammonolysis of Glycosylation Product (6B)

The procedure for the ammonolysis of 6B was based on procedures of several investigators: Wright et al. (1987); Kazimierczuk et al. (1990); Himmelsbach et al. (1984); Trichtinger, Charubala, and Pfeleiderer (1983); and Aerschot et al. (1988). The methods involved use of either concentrated aqueous ammonia in methanol or anhydrous ammonia in methanol with the latter proving more efficient in removing the 3',5' p-toluoyl groups to yield the desired compound 9.

Fig. 16. UV-visible Spectrum (methanol) of Fraction
Containing N^2 -(1-(trans-2-hydroxy-acenaphthenyl))-
 O^6 -p-nitrophenylethyl-9-(2-deoxy-3,5-di-O-p-toluoyl-
 β -D-erythro-pentofuranosyl)purine (8)



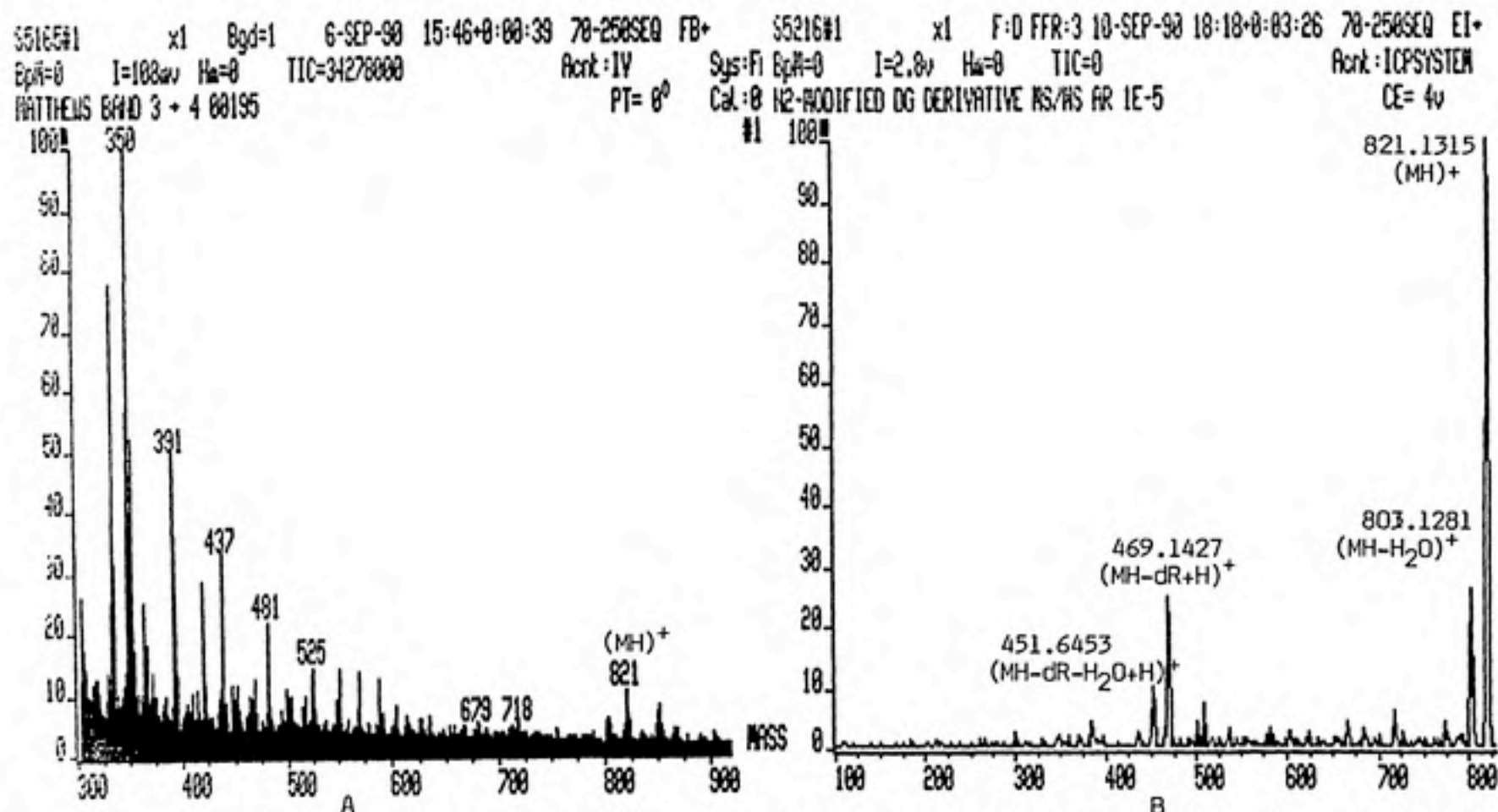


Fig. 17. FAB Mass Spectrum of N^2 -(1-(trans-2-hydroxy-acenaphthenyl))-O⁶-p-nitrophenyl-ethyl-9-(2-deoxy-3,5-di-O-p-toluoyl- β -D-erythro-pentofuranosyl)purine (8)
 (A), Full Scan of Fraction; (B), FAB MS/MS.

The reaction sequence, shown in figure 8, was repeated nine times in order to optimize reaction conditions and to generate sufficient amounts of compound 9 for use in future condensation reactions. Reaction conditions for optimal yield are as follows.

2-Bromo-O⁶-p-nitrophenylethyl-2'-deoxyinosine (9)

Anhydrous ammonia was bubbled through a stirred, cooled (0°C) suspension of compound 6B (40 mg) in methanol (20 mL) until the solution was saturated (approximately 10 min). The mixture was then stoppered and stirred at room temperature until the off-white solid dissolved in the solution (approximately 9 h). The methanol and ammonia were then removed on a rotary evaporator under oil pump vacuum at room temperature.

The clear residual was chromatographed on silica gel preparative TLC plates eluted with 5% methanol in chloroform, and the lowest band ($R_f=0.27$) afforded a clear/white solid, pure compound 9 (15 mg, 56% yield). Additional bands resolved by the prep TLC chromatography gave products identified as NPE-group-deprotected derivatives of 6B, which will be discussed further in Chapter IV.

The structural identity and purity of compound 9 were confirmed by the following characteristics: Melting Point 86-89°C. ¹H NMR (400 MHz, DMSO-d₆) (see figure 18) -
δ 2.29-2.34, 2.62-2.68 (2H, m, H2', H2''); 3.47-3.61 (2H, m,

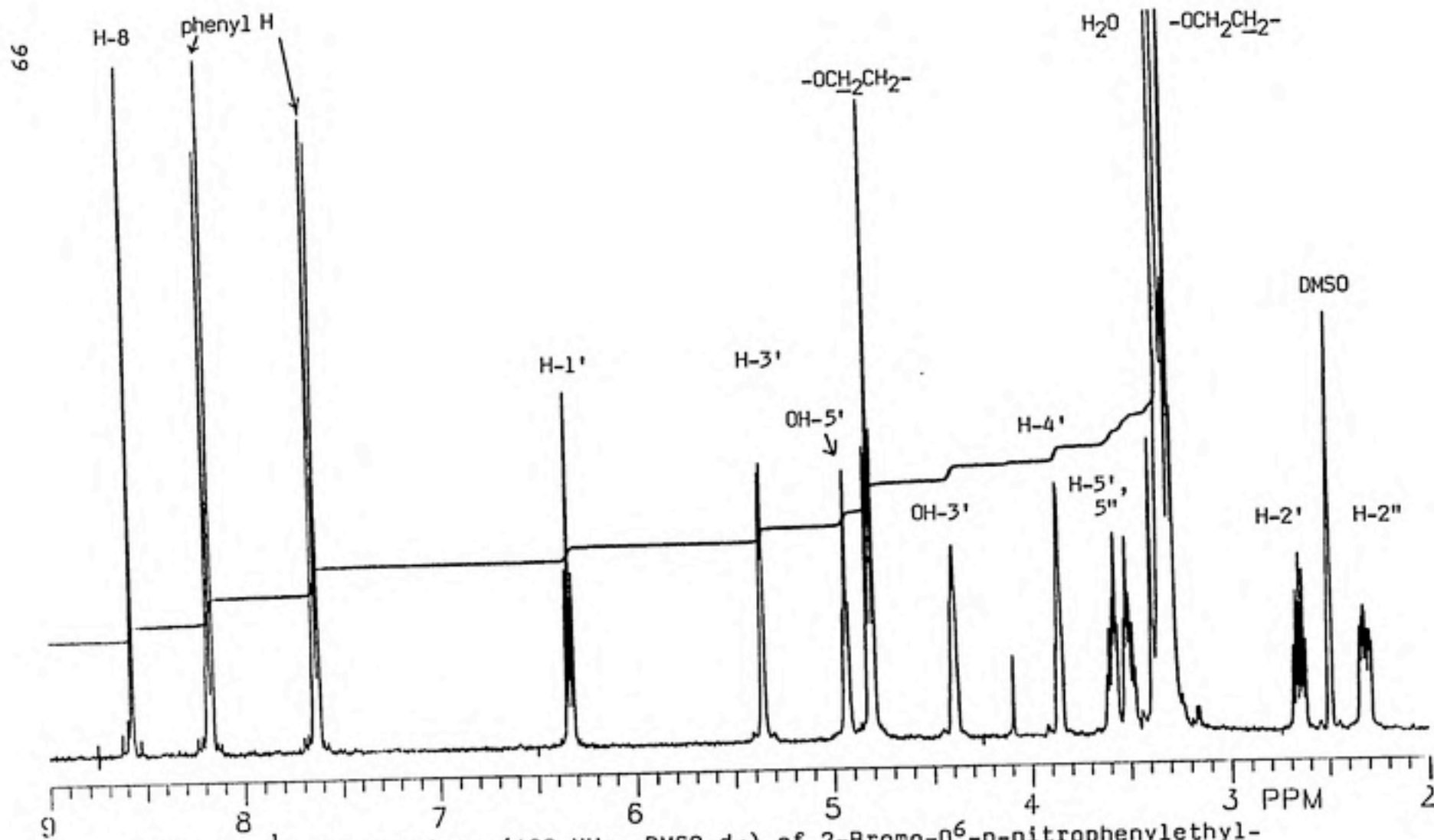


Fig. 18. ¹H NMR Spectrum (400 MHz, DMSO-d₆) of 2-Bromo-O⁶-p-nitrophenylethyl-2'-deoxyinosine (9)

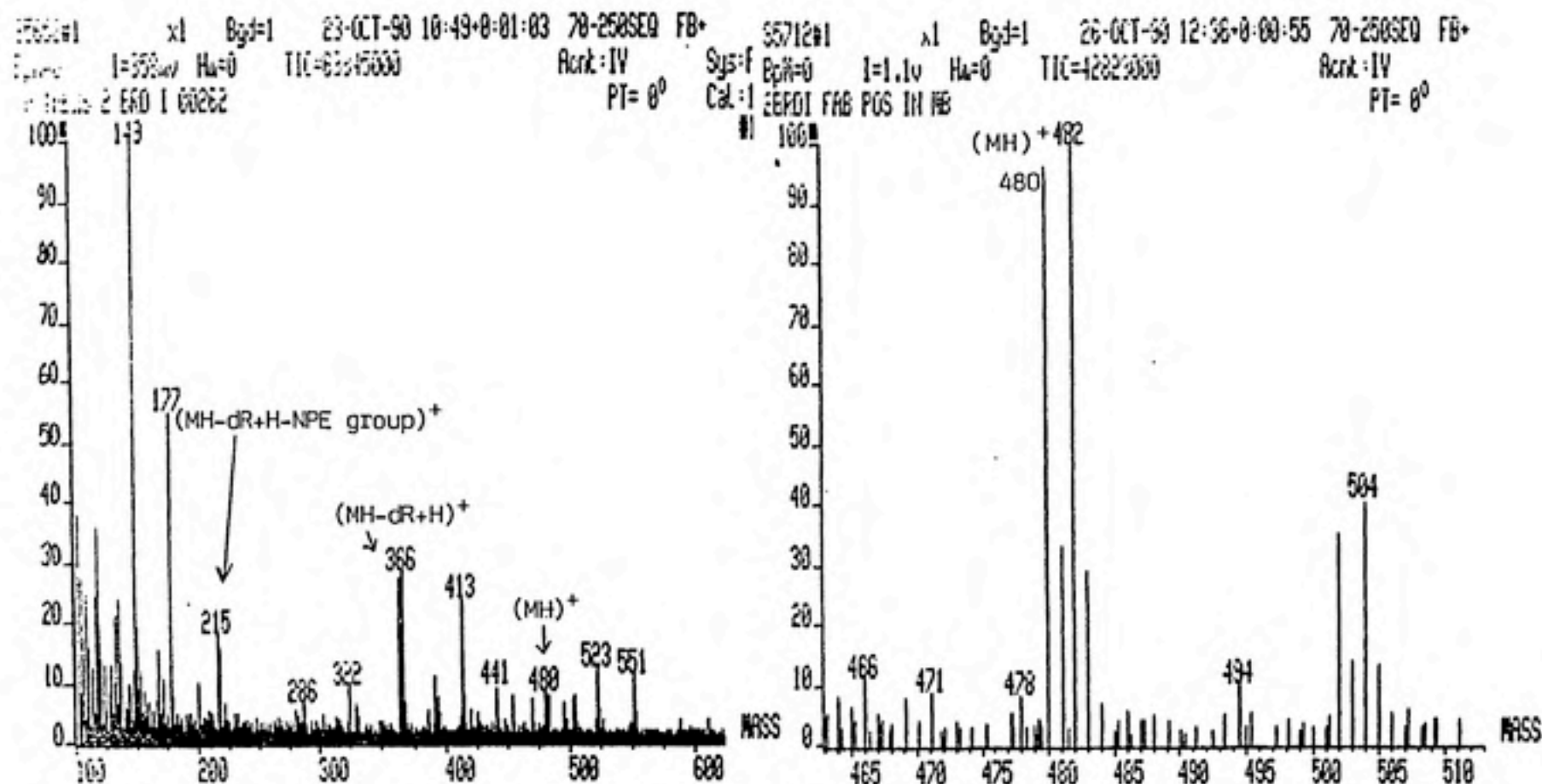


Fig. 19. FAB Mass Spectrum of 2-Bromo-O⁶-p-nitrophenylethyl-2'-deoxyinosine (9).

H5', H5''); 3.84-3.87 (1H, m, H4'); 4.39 (1H, m, OH3'); 4.81 (2H, t, $J_{\text{ave}}=6.55$ Hz, OCH_2CH_2); 4.92-4.94 (1H, m, OH5'); 5.35 (1H, m, H3'); 6.33 (1H, pseudo t, $J_{\text{ave}}=6.65$ Hz, H1'); 7.63, 8.17 (2H each, 2d, $J=8.6$ Hz each, phenyl Hs); 8.57 (1H, s, H8). FAB Mass Spectrum (see figure 19) - m/z 480, 482 (MH^+); 364, 366 ($\text{MH}-\text{dR}+\text{H}^+$); 215, 217 ($\text{MH}-\text{dR}+\text{H}-\text{NPE}$ group) $^+$; Accurate Mass of MH^+ - observed 480.4261, 2.9 ppm difference from calculated value for $\text{C}_{18}\text{H}_{18}\text{O}_6\text{N}_5^{79}\text{Br}_1+\text{H}$.

Condensation of O⁶-NPE-Protected 2-Bromo-2'-deoxyinosine (9) with Cyclopenta-PAH amino alcohol (7)

The second condensation reaction involving 2-bromo-2'-deoxyinosine derivative 9 and the same cyclopenta-PAH derivative 7 (see figure 8 for synthetic scheme) was carried out according to the same published procedures as the first. The reaction steps were as follows.

N²-(1-(trans-2-hydroxy-acenaphthenyl))-O⁶-p-nitrophenylethyl-2'-deoxyquanosine (10)

A mixture of O⁶-NPE-protected 2-BrdI 9 (50 mg, .10 mmol), cyclopenta-PAH amino alcohol 7 (35 mg, .19 mmol), and triethylamine (.014 mL, .10 mmol) in 7 mL methoxyethanol was refluxed under argon with stirring for 26 h. The solvent was removed on a rotary evaporator at 95°C, and the resulting brown syrup was placed on reversed phase preparative TLC plates developed with 80% methanol in water.

Products from the ten resolved bands were screened by UV-visible spectroscopy for the acenaphthene chromophore and then subjected to FAB mass spectrometry, which failed to

detect the MH^+ molecular ion ($m/z=585$) corresponding to compound 10. According to UV-visible and mass spectral behavior, the most promising fraction (band 8, $R_f=0.24$, fluoresced light blue under long wave UV) was selected and purified by HPLC using the following solvent program (% methanol in water at 2 mL/min): 5 min at 70%; 70 to 100% in 20 min; and 100% for 10 min. UV-visible and 1H NMR spectra were recorded for products from the major peaks that eluted after 15 min (3.5 mg) (see figures 20 and 21).

UV-visible spectra (methanol) suggested the presence of the adduct 10 in band 8 (λ_{max} 280, 224 nm; figure 20), and in the 1H NMR spectrum (400 MHz, CD_3OD), both aromatic and deoxyribose proton resonances were observed, further supporting the presence of 10 (figure 21). However, the molecular ion could not be confirmed by FAB MS so additional HPLC was performed on band 8 using the following solvent program: 5 min at 55%; 55 to 85% in 15 min; and 85 to 100% in 10 min. A peak eluting from 28-36 min (2.5 mg of product) gave suggestive UV-visible and 1H NMR spectra (see figures 22 and 23), but it was still not possible to confirm 10 by FAB MS.

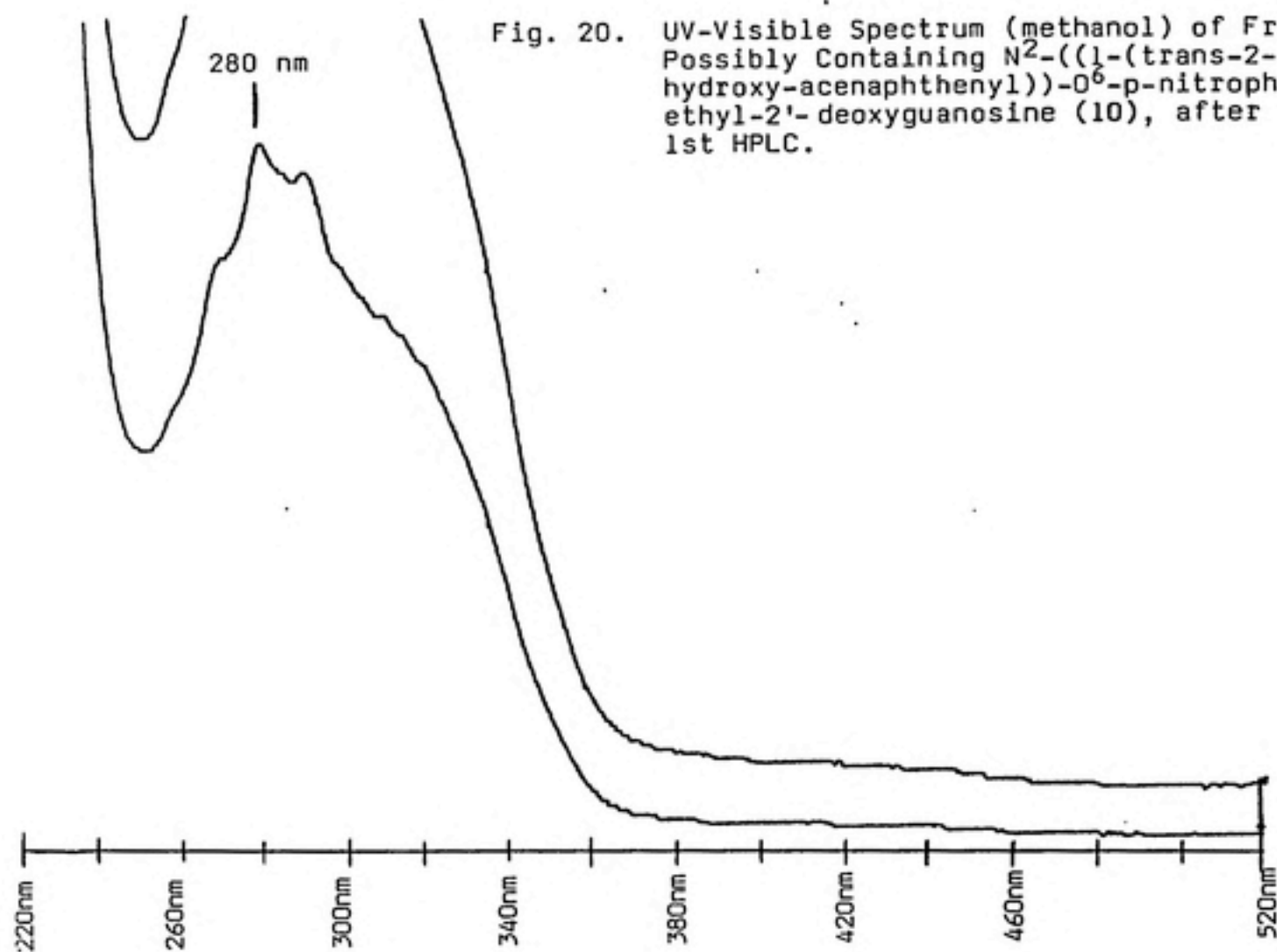


Fig. 20. UV-Visible Spectrum (methanol) of Fraction Possibly Containing N^2 -((1-(trans-2-hydroxy-acenaphthenyl))- O^6 -p-nitrophenyl-ethyl-2'-deoxyguanosine (10), after 1st HPLC.

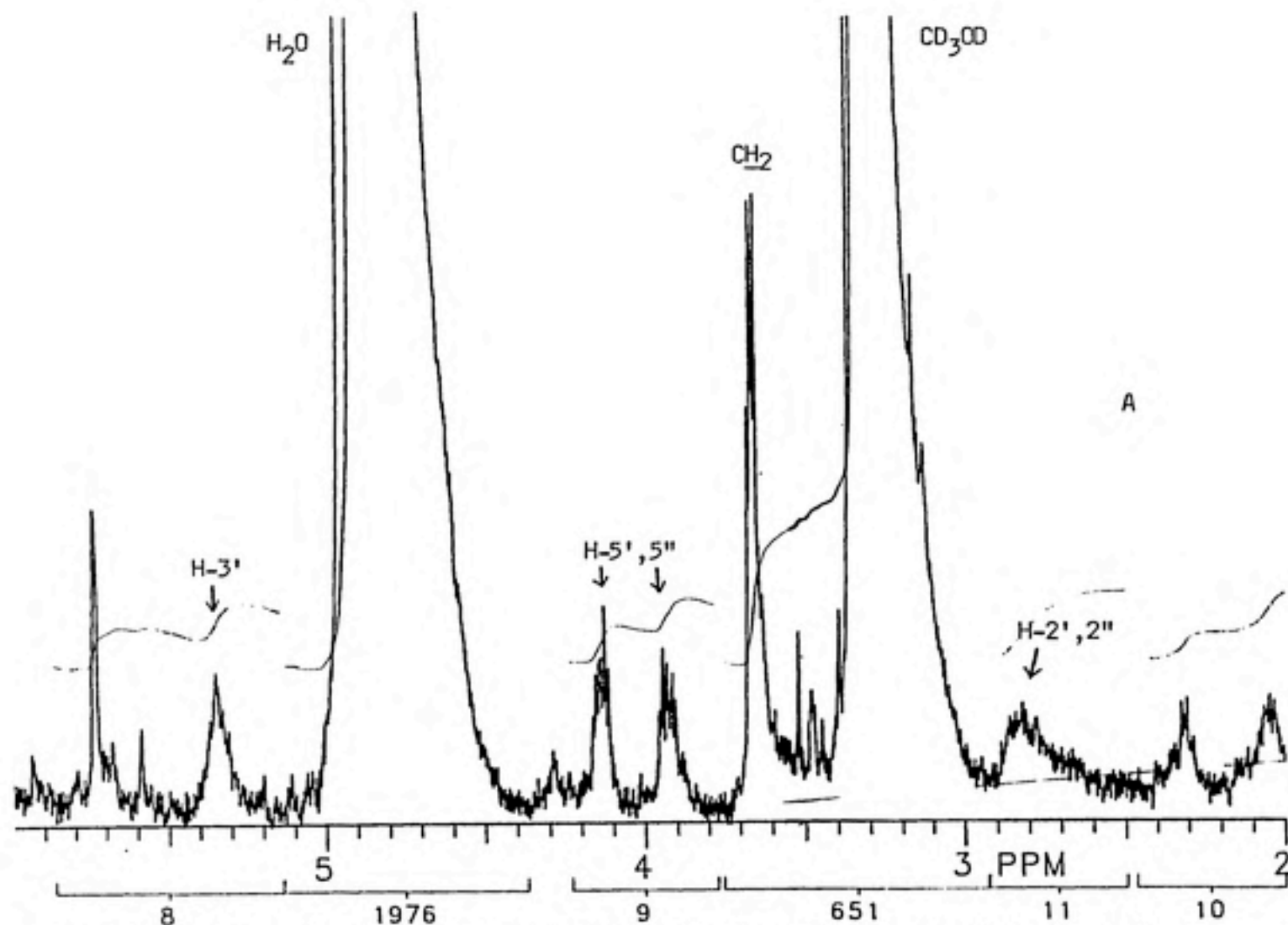


Fig. 21. ^1H NMR Spectrum (400 MHz, CD_3OD) of Fraction Possibly Containing N^2 -(1-(trans-2-hydroxy-acenaphthenyl))- O^6 -p-nitrophenylethyl-2'-deoxyguanosine (10), after 1st HPLC. (A), 2-6 ppm; (B), 6-9 ppm.

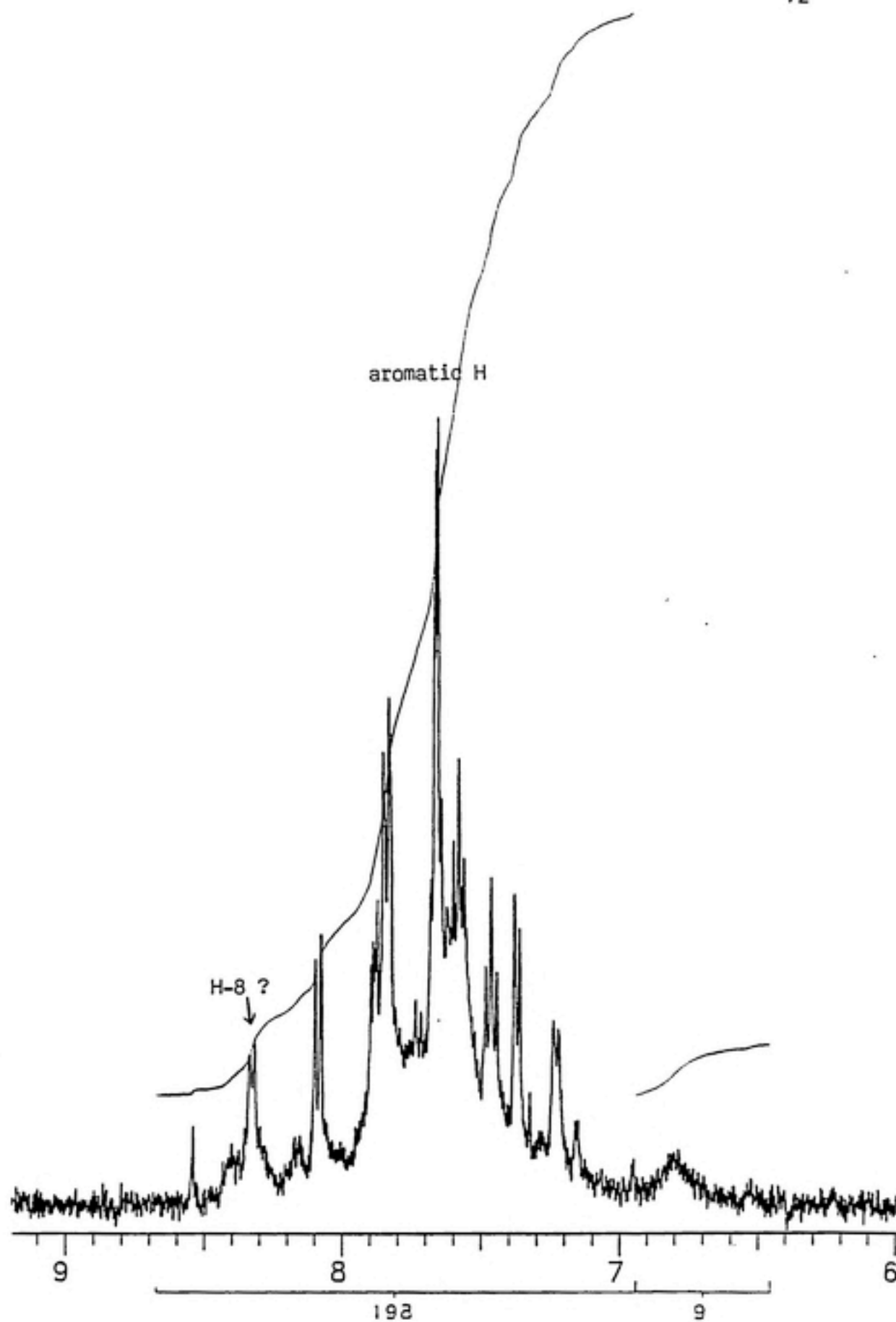
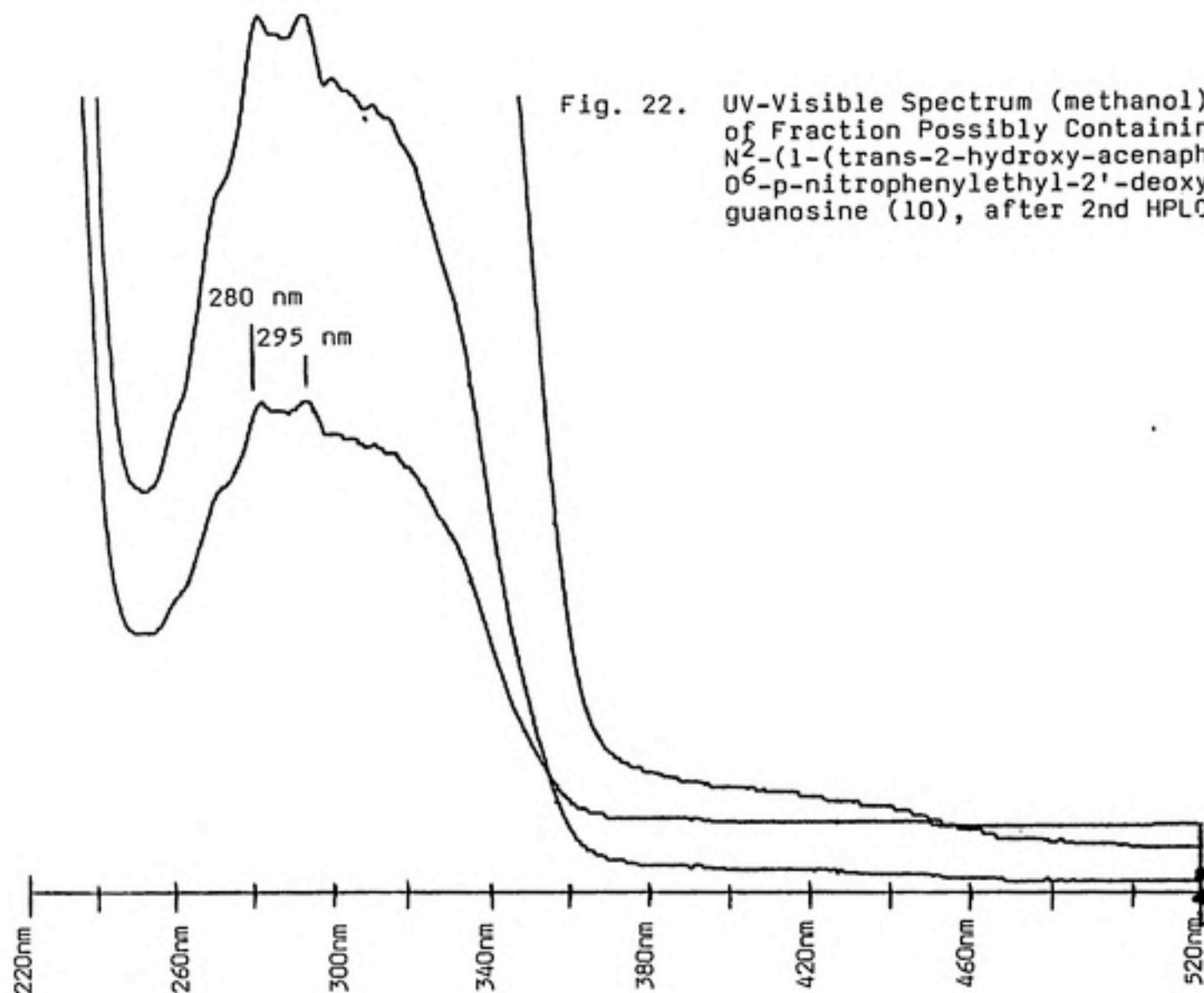


Fig. 21. B.



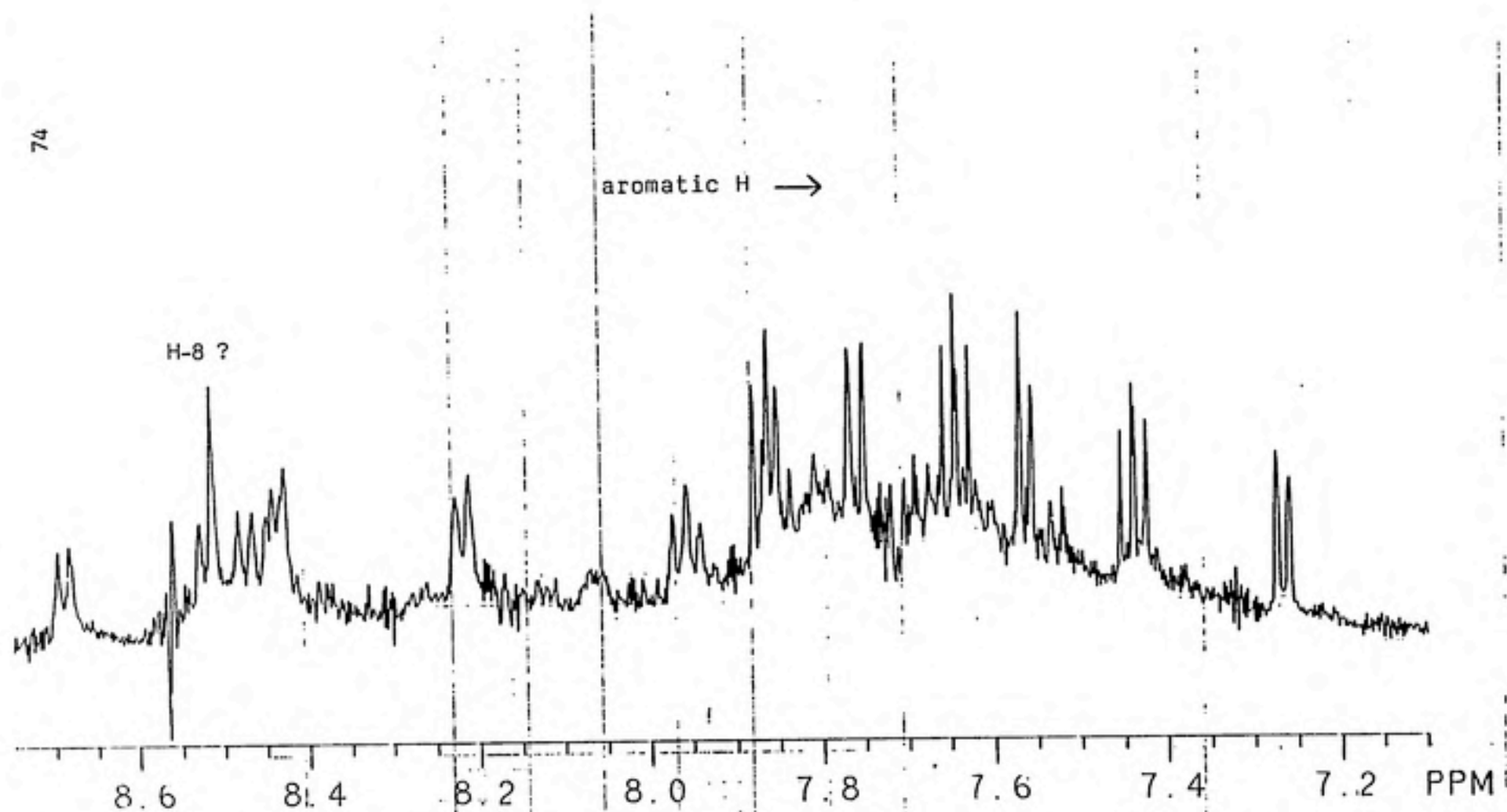


Fig. 23. Partial ^1H NMR Spectrum (500 MHz, DMSO-d_6) of Fraction Possibly Containing N^2 -(1-(trans-2-hydroxy-acenaphthenyl))-O 6 -p-nitrophenylethyl-2'-deoxyguanosine (10), after 2nd HPLC.

CHAPTER IV

RESULTS AND DISCUSSION

Synthesis of O⁶-NPE-Protected 2-Bromohypoxanthine

The synthesis of O⁶-NPE-protected 2-bromohypoxanthine (4) was carried out according to the (somewhat vague) three-step procedure of Raju, Robins, and Vaghefi (1989), who claim that 2-bromo-O⁶-p-nitrophenylethylpurine "can be prepared on a large scale in 75% overall yield, without requiring chromatographic purification in any step during its preparation". However, in the two separate syntheses conducted in this study, modifications were required regarding work-up, and yields were less than published values. See figure 7 for the synthetic scheme.

The first step in the sequence, involving tritylation of 2-bromohypoxanthine (1), proceeded according to the reference (Raju, Robins, and Vaghefi 1989), but the resulting sludgy residue containing compound 2 couldn't be weighed in order to determine the reaction yield (which was reported as being quantitative).

The second step in the sequence, involving introduction of the O⁶-NPE group to generate compound 3, necessitated some variations from referenced procedures. Despite attempts in two separate syntheses, crystallization of tritylated 2-bromo-O⁶-p-nitrophenylethylpurine (3) from 2-

propanol failed to give pure 3 but rather a precipitate shown by ^1H NMR analysis (400 MHz, CDCl_3) to contain phenyl impurities (possibly triphenylphosphine oxide side product). This impure reaction mixture was used in the next step, involving detritylation with 80% acetic acid, but the reaction failed to generate compound 4. Hence, column chromatography of the reaction mixture was required in order to obtain pure compound 3 prior to proceeding in the synthesis. Best results in separating the N-9 and N-7 isomers of 3 from impurities were obtained by initially using benzene:chloroform (1:3) as eluant followed by pure chloroform after appearance of the fraction containing the N-9 isomer of 3. The N-7 isomer of 3 eluted later.

The structure and purity of the N-9 and N-7 isomers of 3 were confirmed by their ^1H NMR spectra (400 MHz, CDCl_3) (see figure 9), and spectra agreed with all proton signals reported by Raju, Robins, and Vaghefi (1989) for the N-9 isomer, including two triplets for the methylene protons on NPE group, two doublets for phenyl protons on NPE group, a multiplet for the trityl group, and a singlet for H-8 of the purine. Integration of the NMR signals verified the proper number of protons. ^1H NMR signals for the N-9 isomer of 3 were shifted downfield from the corresponding N-7 isomer signals, as illustrated by the following examples: for the N-9 isomer - singlet for H-8, $\delta = 7.92$ ppm, and downfield triplet for CH_2 , $\delta = 4.79$ ppm; for the N-7 isomer - singlet

for H-8, $\delta = 7.87$ ppm, and corresponding downfield triplet for CH_2 $\delta = 4.11$ ppm.

The yield of compound 3 (combined N-9 and N-7 isomers) from compound 1 was 28% in the first synthesis and 14% in the second. However, in the second synthesis some of the N-7 isomer of 3 remained on the column and was not included in the yield calculation. Since a larger yield of 85% was reported for this step (Raju, Robins, and Vaghefi 1989), there is some discrepancy observed in this study, possibly resulting from the crystallization procedure.

In the third step of the sequence, involving detritylation of 3 with 80% acetic acid to generate compound 4, a novel work-up technique was successfully employed to obtain pure 4. This involved (2 X) trituration of the reaction product with chloroform to give a precipitate, determined to be pure 4. Chloroform was chosen because compound 3 and side products (trityl alcohol and trityl acetate) are highly soluble in this solvent while compound 4 shows little or no solubility in it.

The yields of 4 from 3 were 85% and 84% in the separate syntheses, close to the reported quantitative yield (Raju, Robins, and Vaghefi 1989). However, in this study the overall yields of 4 from 1 were only 24% and 12% in the separate syntheses in contrast to the published value of "overall 75% yield" (Raju, Robins, and Vaghefi 1989).

The ^1H NMR spectrum (400 MHz, DMSO-d_6) of compound 4 (see figure 10) concurred with published data from Raju,

Robins, and Vaghefi (1989) and showed the following characteristic signals: one triplet for methylene protons on NPE group (other triplet was obscured by a peak from water), two doublets for phenyl Hs on the NPE group, singlet for H-8 on purine, and singlet for H-9 on purine (shown at 13.5 ppm on spectra with spanning 0-20 ppm).

Lewis Acid Catalyzed Glycosylation of O⁶-NPE-Protected
2-Bromohypoxanthine

The glycosylation of O⁶-NPE-protected 2-bromohypoxanthine (4) with the deoxyribose derivative (5A) (see figure 7 for synthetic scheme) involved use of a silylating agent, BSA, and the Lewis acid catalyst, TMSOTf. Methods employed by Raju, Robins, and Vaghefi (1989) and Wright and Dudycz (1984) were followed for the reaction procedures and work-up.

Glycosylation products that eluted from prep TLC on silica gel were (first) a mixture of N-9 α and β isomers (R_f = 0.4) and (later) a mixture of N-7 α and β isomers (R_f =0.2) with the relative amounts of N-9:N-7 equal to approximately 4:1 and β anomer/ α anomer approximately 45% /55%. Yield of the N-9 α and β glycosylation mixture was acceptable at 56%. However, several attempts at separating the anomers by various combinations of prep TLC plate thicknesses and eluant mixtures proved unsuccessful.

Raju, Robins, and Vaghefi (1989) reported that their Lewis acid catalyzed glycosylation reaction between compound

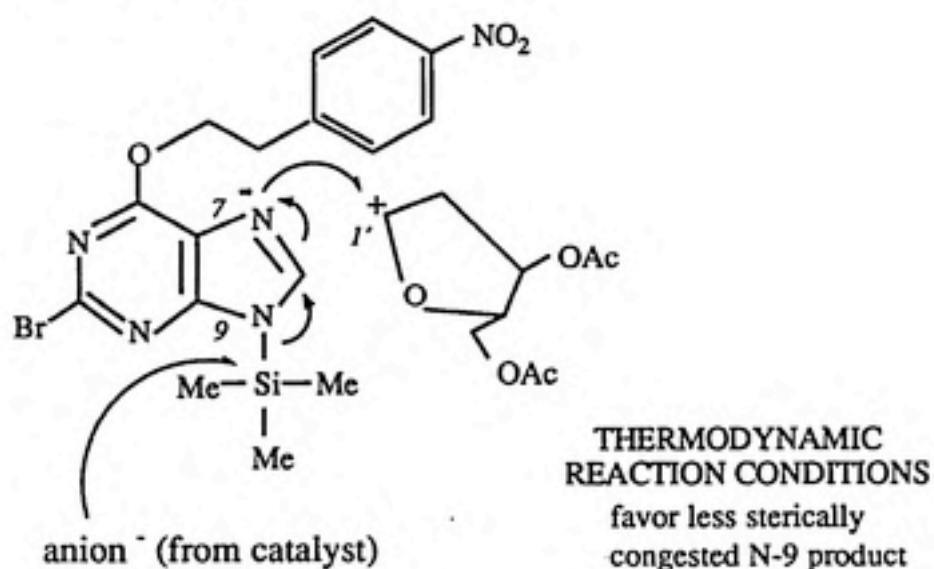
4 and 1,2,3,5-tetraacetylribose gave 95% yield of the N-9 β isomer with a ratio of 20:1 for N-9:N-7.

The mechanism of the S_N1 Lewis acid catalyzed glycosylation reaction between the sugar cation and nucleophilic purine site (N-7/N-9) is discussed in the literature by Niedballa and Vorbruggen (1974) and Dempcy and Skibo (1990). As shown in figure 24(A), initial silylation should occur at the N-9 position rather than the N-7 position due to steric effects from the NPE group. Following removal of the silyl group by the catalyst anion, ribosylation with the sugar cation (at H-1') would afford the N-7 isomer as the major kinetic product (see figure 24(A)). However, under thermodynamic reaction conditions (long reaction time in a polar solvent) the more stable, less sterically congested N-9 isomer should be favored.

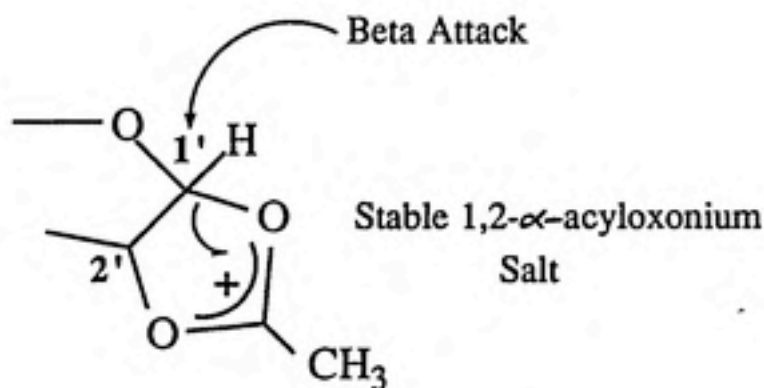
Niedballa and Vorbruggen (1974) investigated these "silyl" glycosylation reactions extensively. They detected only the β anomer of the glycosylation products in the presence of a 2 α -acyloxy (e.g., -OAcetyl group) substituent in the sugar moiety. On the other hand, they found both anomeric (α and β) glycosylation products to be formed in the case of acylated 2'-deoxyribose derivatives.

In fact, Niedballa and Vorbruggen (1974) reacted a silylated pyrimidine with chlorofuranose (compound 5B in figure 8) and "in a number of experiments found a nearly constant ratio of anomers $\alpha/\beta \sim 1$ which apparently could not be influenced by variation of the reaction conditions".

KINETIC PRODUCT



A



B

Fig. 24. Reaction Mechanism of Lewis Acid Catalyzed Glycosylation. (A), Under Kinetic Conditions- N-7 Isomer as Major Product (modified from Dempcy and Skibo 1991, p. 779) ; (B), With 2- α -Acyloxy Sugars - β Anomer As Exclusive Product (modified from Niedballa and Vorbruggen 1974, p. 3657).

They further explained the exclusive formation of β anomers in the case of 2- α -acyloxy sugars as a result of formation of the rather stable 1,2- α -acyloxonium salt. This cyclic sugar cation hinders α attack by the purine nucleophile during the glycosylation reaction, as shown in figure 24(B).

Thus, the reaction mechanism proposed in the literature explains the formation of α anomers in the Lewis acid catalyzed glycosylation reaction carried out in this study. However, from Raju and co-workers' report (1989), one would expect much higher ratios of N-9/N-7 glycosylation isomers than were observed in this study. This suggests that the p-nitrophenylethyl protecting group on O-6 of the purine may not be as effective in sterically hindering N-7 glycosylation reaction as Raju, Robins, and Vaghefi (1989) claim.

In this study, distinction of the N-9 glycosylation isomers from the N-7 isomers was made by analogy to ^1H NMR data reported for other 2'-deoxynucleoside glycosylation products, including reports by Wright and Dudycz (1984), Kazimierczuk et al. (1984), and Kazimierczuk et al. (1990). In all of these investigations, the N-7 isomer (α or β anomer) was differentiated from the N-9 isomer (α or β anomer) by key spectral features: the H-8 signal of the N-7 isomers is shifted downfield from both the phenyl H doublets and the H-8 signal of the N-9 isomers; and the H-4', H-5', and H-5'' signals of the N-7 isomers overlap, compared to the

more resolved signals of the N-9 isomers. In addition, the α anomers can be distinguished from the β anomers by the fact that the H-1' signal of the β anomers is a pseudo-triplet, while the H-1' signal of the α anomers is a broad "pseudo" doublet. The ^1H NMR spectrum of the α and β anomeric mixture of N-9 glycosylation product 6A (figure 11) shows the expected behavior of the NMR signals including: an upfield shift of singlet for H-8; resolution of multiplet signals for H-4', H-5', and H-5"; and both a pseudo-triplet signal for H-1' (β anomer) and a broad doublet signal for H-1' (α anomer).

Sodium Salt Glycosylation of O⁶-NPE-Protected
2-Bromohypoxanthine

Since the products of Lewis acid catalyzed glycosylation of compound 4 with deoxyribose derivative 5A could not be separated to give the desired pure N-9 β glycosylation product, another glycosylation reaction mechanism was sought that could ensure separable glycosylation isomers, preferably with the N-9 β anomer as the predominant product. Published reports (Niedballa and Vorbruggen 1974) and unpublished results in Dr. Gold's lab indicated that the Lewis acid catalyzed glycosylation of silylated purine derivatives with chlorofuranose (5B) produced mixtures of α and β anomers not readily separable.

Thus, in this study it was decided to try the sodium hydride catalyzed glycosylation of compound 4 with chlorofuranose 5B (see figure 8 for synthetic scheme). The

"sodium salt glycosylation" method has been reported to yield only β anomers, with the 9- β isomer as major product and the 7- β isomer as minor product (Kazimierczuk et al. 1984, 1990). The reaction mechanism is thought to involve an S_N2 attack of the anionic purine nitrogen (N-7/N-9) on the α -anomer of the 1-chloro sugar (5B) (Kazimierczuk et al. 1984, 1990).

In this study both sodium salt glycosylations of 4 with 5B gave N-9 β and N-7 β isomeric products. These glycosylation products were easily separated and purified by column chromatography on silica gel by utilizing an initial eluant of 4% acetone in toluene to elute the N-9 β isomer and later 6% to 10% acetone in toluene to elute the N-7 β isomer. No N-7 or N-9 α anomers were detected from the reaction.

The yields of the desired N-9 β isomer (compound 6B) were moderate and consistent at 39% and 34% for two separate reactions. The relative amounts of N-9 β isomer/N-7 β isomer were also consistent at 42% /58% and 44% /56%, but were in strong contrast to the expected results.

Introduction of the bulky p-nitrophenylethyl group at O-6 of the purine was expected to favor formation of N-9 β isomeric product over N-7 β product, in accord with the report by Raju, Robins, and Vaghefi (1989) for the Lewis acid catalyzed glycosylation of compound 4. Other investigators (Kazimierczuk et al. 1984, 1990) have also reported results of sodium salt glycosylations of 2,6-disubstituted purines,

which show the expected predominance of N-9 β glycosylation products over N-7 β isomeric products as follows (N-9 β /N-7 β): for 2,6-dibromo - 88% /12% ; for 2,6-bismethylthio - 76% /24% ; and for 2,6-dichloro - 82% /18%. For 6-chloropurine the N-9 β /N-7 β ratio was 84% /16%.

Based on results of both the Lewis acid catalyzed and sodium hydride catalyzed glycosylations in this study, the O⁶-NPE protecting group does not seem to be providing suitable steric hindrance for significantly limiting glycosylation at the N-7 position of the purine. This might be due, in part, to the fact that the p-nitrophenylethoxy group could rotate about the C-6 - O-6 single bond, which would consequently reduce its steric interference with attack at N-7. For the sodium salt glycosylations, the "large" bromine group on the 2 position of the purine may be more effective in directing N-7 (vs. N-9) glycosylation than the O⁶-NPE protecting group is in hindering N-7 reaction. This suggestion is supported by results of this study, in which the N-7 isomeric product predominated slightly in the two sodium salt glycosylation reactions.

Confirmation of the structure and purity of the N-9 β glycosylation isomer (6B) and the N-7 β isomer was obtained from their ¹H NMR spectra and mass spectrum (for 6B) (see figures 13, 14, and 15). As expected, the NMR signal for H-8 of the N-7 β glycosylation product was downfield (δ =8.37 ppm) from the H-8 signal of the N-9 β product (6B) (δ =8.07 ppm), and the multiplet signal for H-4',5', and 5" of the

N-7 β isomer strongly overlapped ($\delta=4.64-4.70$ ppm) compared to the corresponding multiplet signal for the N-9 β isomer (partially resolved: $\delta=4.64-4.69$ ppm; $4.74-4.77$ ppm).

The NMR spectrum of compound 6B (see figure 13) showed all of the signals expected for the β anomer of a protected 2'-deoxynucleoside, including the following: two singlets for methyl protons of the p-toluoyl groups; a multiplet for H-2',2"; two triplets for the methylene protons of the NPE group; a multiplet for H-4',5',5"; a multiplet for H-3'; a pseudotriplet for H-1' (characteristic of β anomers); six doublets (12 H total) for phenyl Hs on NPE and p-toluoyl groups; and an "upfield" singlet for H-8 of the purine.

The FAB mass spectrum of compound 6B (see figure 14) showed characteristic ions for loss of bromine and loss of the ribosyl derivative as well as the expected molecular ion (MH^+ $m/z=716,718$). Also, an accurate mass measurement of the molecular ion confirmed the elemental composition of $C_{34}H_{30}O_8N_5^{79}Br_1$.

Condensation of Glycosylation Product with Cyclopenta-PAH amino alcohol

Since a 2-bromo-2'-deoxyinosine derivative (compound 6B) was successfully synthesized in this study, its usefulness as a precursor to N²-modified 2'-deoxyguanosine (a protected DNA adduct of a CP-PAH) could be tested. Thus, the coupling of compound 6B with enantiomeric 1-amino-2-hydroxy cyclopenta-PAH (7), was attempted (see figure 8 for synthetic scheme).

Trans acenaphthene 1-amino-2-ol (7) was available from earlier work by Bartczak (1989) in which the condensation with 6-chloropurine-9- β -D-ribofuranose had been accomplished to generate the diastereomeric N⁶-adenosine adducts (Bartczak et al. 1989). Refluxing ethanol was used as the solvent for the condensation reaction in that study. Other condensation reactions (Hildebrand et al. 1990; Wright and Dudycz 1984) between 2-bromohypoxanthine and amines employed refluxing 2-methoxyethanol.

In this study refluxing 2-methoxyethanol (B.Pt.=124°C) was chosen for initial attempts at the condensation of compound 6B with 7. A few drops of triethylamine were added to neutralize HBr formed during the substitution. The reaction was monitored by analytical TLC (silica gel 0.2 mm layer sheets) until no changes could be observed, during which time the solution changed in color from light yellow to green to light brown. After 24 h, the reaction appeared complete and was terminated. Work-up by chromatography on silica gel prep TLC plates gave ten reaction products resolved in separate bands. The products were characterized by fluorescence (under long wave UV), UV-visible spectroscopy, and FAB mass spectrometry (in a full scan mode).

In prior unpublished laboratory results, the UV-visible spectrum of an acenaphthene-deoxyadenosine (dA) adduct had been recorded and showed a characteristic shape with λ_{max} at 223 nm and 275 nm. From the condensation reaction in

this study, product from the prep TLC bands that chromatographed directly beneath starting material 6B showed a UV-vis spectrum similar to that of the acenaphthene chromophore (see figure 16) and also fluoresced light blue under long wave UV as expected. FAB mass spectrometry on this reaction product confirmed the presence of the protected acenaphthene-deoxyguanosine derivative (8), as shown by its molecular ion (MH^+ $m/z=821$) (see figure 17(A)). As shown in figure 17(B), further support for the adduct was obtained by FAB MS/MS on this molecular ion which gave fragments for loss of water (from the acenaphthene alcohol moiety) and loss of the protected ribosyl derivative from both the molecular ion and dehydrated fragment.

The band containing compound 8 yielded 3.5 mg of product giving a yield of ~5%. Because the cyclopenta-PAH derivative (7) was an enantiomeric mixture, coupling with the β -ribose would be expected to yield diastereomers of 8; however, they were not resolved by TLC work-up nor were the spectroscopic techniques applied to characterization of structure appropriate to demonstrate the mixture.

Although the yield was low (most likely due to the instability of compound 7) in this initial experiment, the condensation demonstrates that the 2-BrdI derivative (6B) is a suitable precursor for generating target N^2 -deoxyguanosine adducts. An attempt was made to improve the yield of the coupling reaction by removing the bulky *p*-toluoyl protecting groups of compound 6B.

Ammonolysis Reactions on Glycosylation Product

Two types of ammonolysis reactions for deblocking 3' and 5' acyl groups of the deoxyribose derivative have been reported (Trichtinger, Charubala, and Pfeleiderer 1983; Himmelsbach et al. 1984; Wright et al. 1987; Aerschot et al. 1988; and Kazimierczuk et al. 1990). They are use of: (1) concentrated aqueous ammonia in methanol, and (2) anhydrous ammonia in methanol.

In this study both ammonolysis procedures were used on compound 6B in order to generate O⁶-NPE-protected 2-bromo-2'-deoxyinosine (compound 9) (see figure 8 for synthetic scheme). After several repetitions with varying conditions (namely, length of reaction), the methanolic/anhydrous ammonia method gave desired product 9 in good yields ranging from 51% - 63%. Optimal yields were achieved when the saturated methanolic ammonia solution with 6B was allowed to stir at room temperature until the white solid starting material completely dissolved. Reaction time for 40 mg of 6B was approximately 9 hours.

During all of the ammonolysis reactions conducted in this study, numerous side-products were observed on prep TLC, which were identified by ¹H NMR spectroscopy or mass spectrometry. In two initial anhydrous ammonia/methanol reactions which were allowed to stir overnight (19-21 h), five products were obtained to which the following structures have been assigned: band 1, p-toluoyl amide;

band 2, 2-bromo-2'-deoxyadenosine; band 3, p-nitrophenylethanol; band 3, a 2-bromo-2'-deoxyadenosine derivative in which the 3' p-toluoyl group remained; and band 4, the desired compound 9.

As shown in figure 25, the ^1H NMR spectrum (400 MHz, DMSO- d_6) of the side-product from band 2 matches signals expected for 2-bromo-2'-deoxyadenosine (see NMR data from Wright et al. 1987; Kazimierczuk et al. 1990: sharp singlet at 8.33 ppm for H-8, broad singlet at 7.83 ppm for NH_2 , a pseudo-triplet at 6.24 ppm for H-1', and the remaining signals corresponding to deoxyribose protons). The NMR spectrum clearly shows absence of the p-nitrophenylethyl group for this product by lack of phenyl proton doublets and methylene proton triplets. It was not possible to obtain confirmatory mass spectral evidence; however, during preparation of this report it was discovered that a modification in standard technique was required in order to obtain mass spectra of bromo-9-ribosylpurines. It is not possible to repeat spectroscopic analysis on side-products from the ammonolysis reactions because of insufficient amounts of remaining products.

Material from band 3 appeared to contain a mixture of two products - p-nitrophenylethanol and a 2-bromo-2'-deoxyadenosine derivative with p-toluoyl group intact at the 3' position. This suggestion is based upon the ^1H NMR spectrum (400 MHz, DMSO- d_6) shown in figure 26.

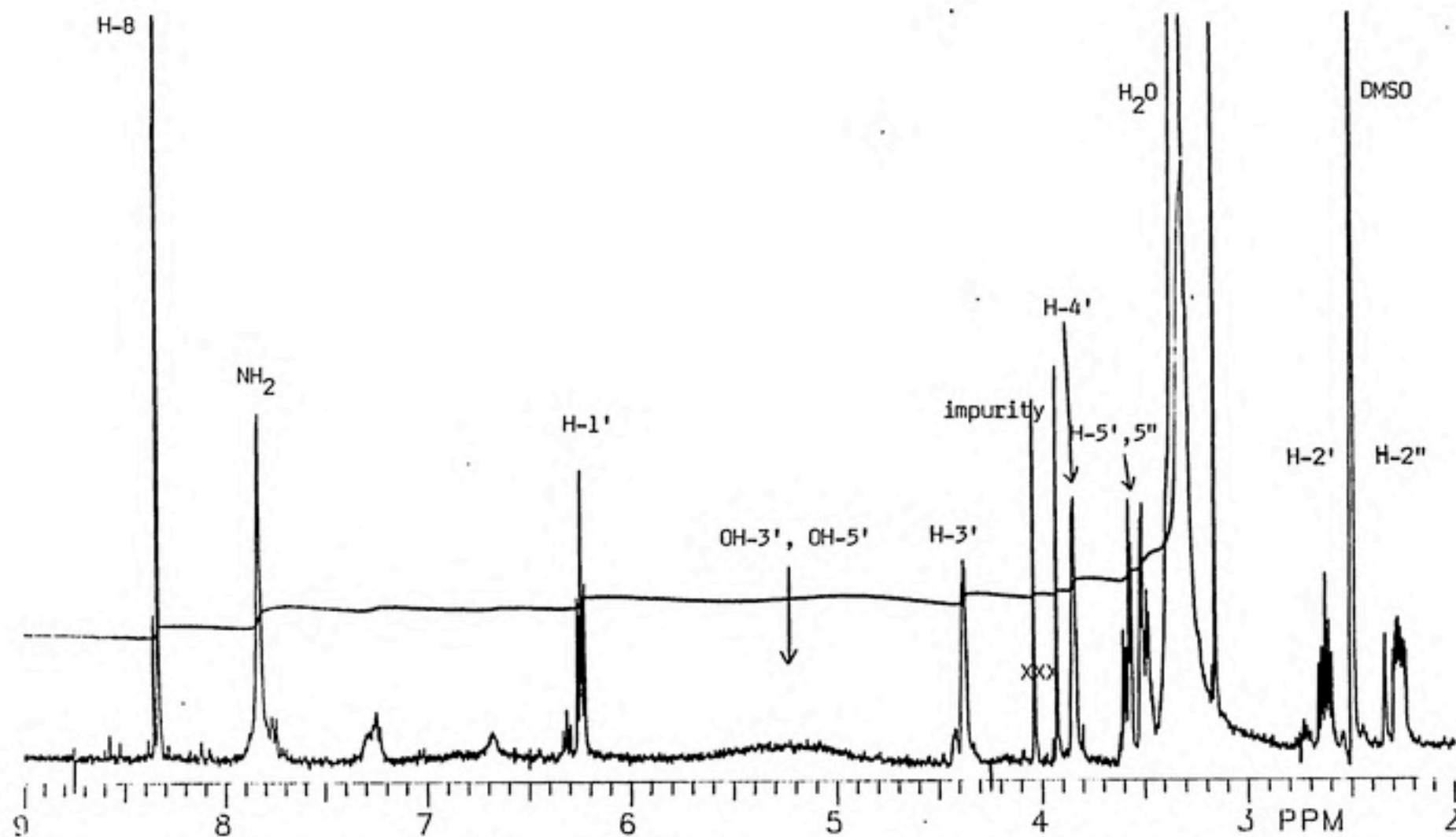


Fig. 25. ^1H NMR Spectrum (400 MHz, DMSO-d_6) of Side-Product in Ammonolysis Rxn - Possibly 2-Bromo-2'-deoxyadenosine.

Integration of the NMR signals in figure 26 shows that the phenyl H doublets at 7.50 ppm and 8.14 ppm are in 1:1 ratio with triplets at 2.85 ppm and 3.65 ppm, but are not in a reasonable ratio with any signals for the deoxynucleoside. Therefore, this product must result from p-nitrophenylethanol generated by partial deprotection of O⁶, and the NMR signals for this alcohol side-product do match the NMR spectrum (figure 28) subsequently obtained for pure NPE-alcohol.

The NMR spectrum in figure 26 also shows signals expected for a deoxynucleoside without the O-6 protecting group but possessing one p-toluoyl group, as evidenced by two phenyl H doublets at 7.24 ppm and 7.76 ppm, the absence of the expected triplets in 1:1 integral ratio, and by the presence of deoxyribose proton signals in the correct integral ratios. Since the multiplet signal for H-5',5" (3.49-3.61 ppm) concurs with that of 2-BrdI (figure 18) and does not show a downfield shift, the p-toluoyl group is assigned to the 3' position of the deoxyribose. With a singlet resonance at 7.89 ppm consistent with presence of an amino group, the second product's NMR spectrum is in accord with a derivative of 2-bromo-2'-deoxyadenosine in which a p-toluoyl group remains on the 3' position.

A confirmatory mass spectrum of the 2-BrdA derivative could not be obtained; however, in view of the modified technique discussed above, this result is inconclusive. The presence of p-nitrophenylethanol as a side-product was

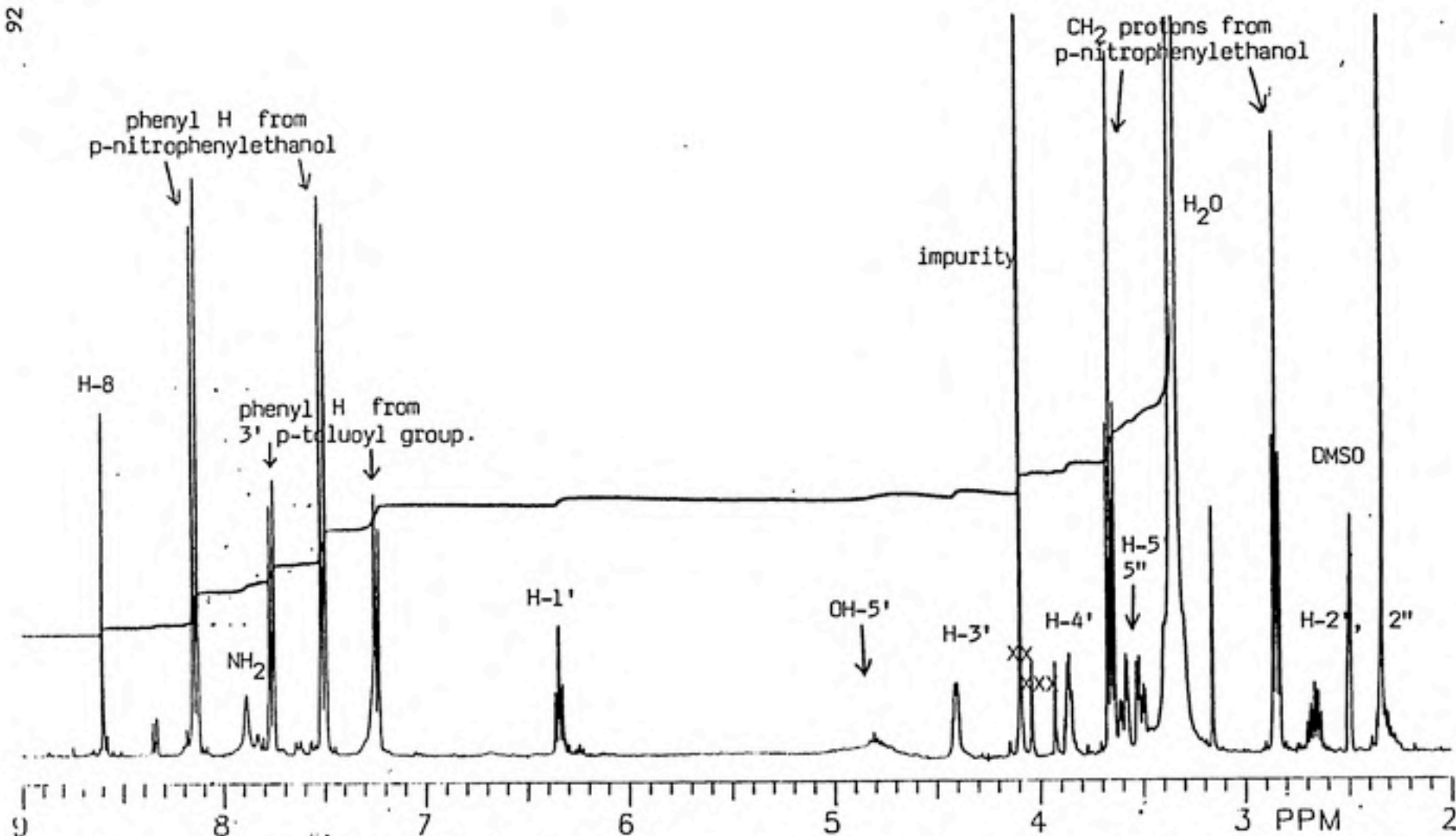


Fig. 26. ¹H NMR Spectrum (400 MHz, DMSO-d₆) of Side-Product in Ammonolysis Rxn - Possibly a 2-Bromo-2'-deoxyadenosine Derivative.

verified by its mass spectrum (EI mode) (see figure 27) which shows the expected molecular ion (M^+ $m/z=167$) plus ions for loss of formaldehyde and loss of water.

In subsequent anhydrous ammonia/methanol reactions which were terminated upon dissolving of the starting material (~ 9 h), at least five products were obtained which were identified as the following: band 1, starting material 6B; band 2, 5'-deblocked 6B; band 3, p-nitrophenylethanol (minor); band 4, 3'-deblocked starting material 6B; and band 5, desired compound 9. The identity of p-nitrophenylethanol was established by its mass spectrum (identical to earlier spectrum) as well as an ^1H NMR spectrum (400 MHz, DMSO- d_6) (see figure 28) that showed signals expected for the phenyl Hs (two doublets), methylene protons (two triplets), and hydroxyl group (broad singlet) in the proper integral ratios.

In one aqueous ammonia (28 - 30% NH_3)/methanol reaction, side-products including p-toluoyl amide, 2-bromo-2'-deoxyadenosine, a 2-BrdA derivative in which the 3' p-toluoyl group remained, and p-nitrophenylethanol were formed, identified by their ^1H NMR spectra and (for NPE-alcohol and p-toluoyl amide) mass spectra.

Thus, in the ammonolysis reactions carried out in this study, some displacement of the alkylated O-6 by ammonia can be inferred by the presence of p-nitrophenylethanol, 2-bromo-2'-deoxyadenosine, and the 3'-protected derivative as side-products. However, the published reports claim that

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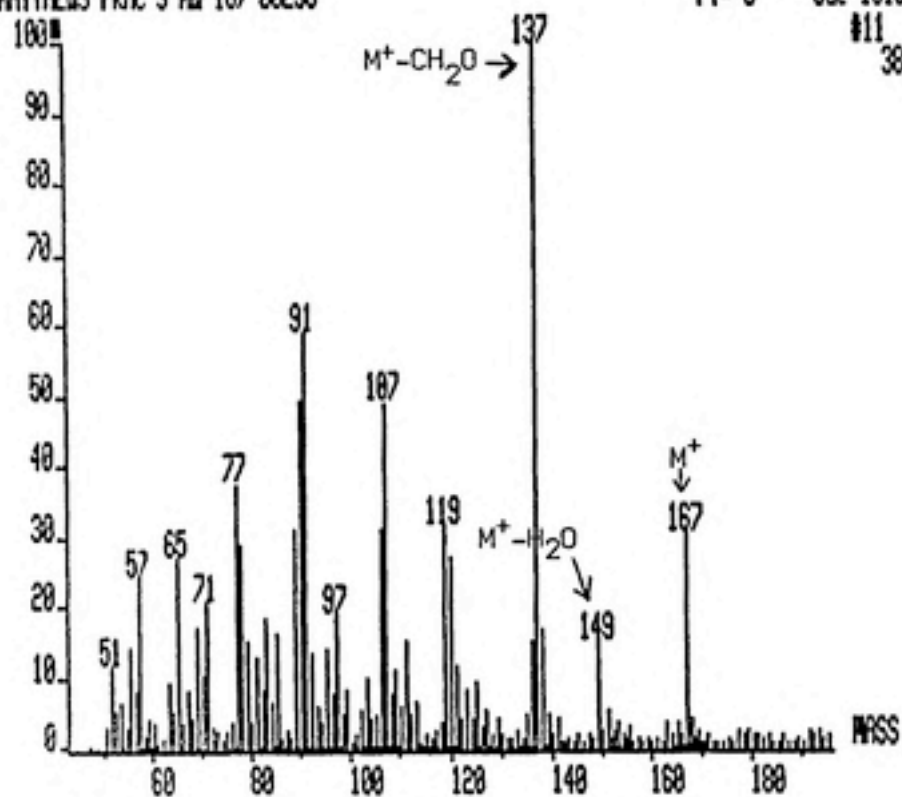
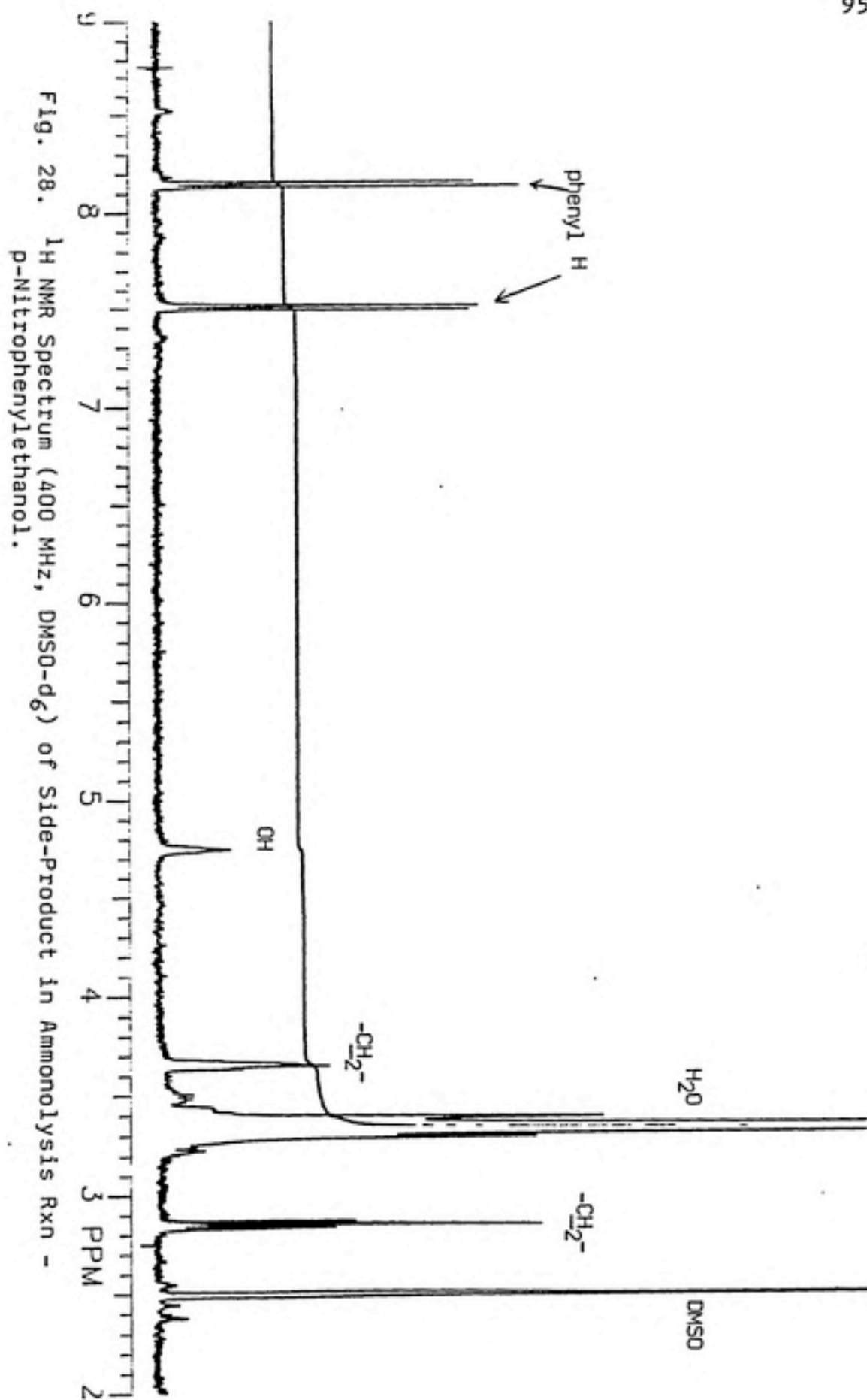


Fig. 27. Mass Spectrum (EI Mode) of Side-Product in Ammonolysis Rxn - p-Nitrophenylethanol.



the O-6 NPE blocking group "is stable against ammonia and triethylamine in methanol, dioxane, and water but can be cleaved quantitatively to regenerate the starting materials by DBN or DBU in aprotic solvents" (Himmelsbach et al. 1984).

Trichtinger, Charubala, and Pfleiderer (1983) also report that prolonged treatment of an N²-acylated, O⁶-NPE protected deoxyguanosine with concentrated ammonia in methanol "cleaves the N²-acyl group without harming the O⁶ blocking group". Aerschot et al. (1988) mention that "MeOH saturated with ammonia hardly caused any deprotection" regarding removal of the O-6 NPE group.

The structural identity and purity of compound 9 were confirmed by ¹H NMR and mass spectra, shown in figures 18 and 19. The NMR spectrum showed all signals expected for the O⁶-NPE-protected purine base coupled at N-9 to 2'-deoxyribose in a β anomer configuration. The FAB mass spectrum showed the expected molecular ions (MH⁺ m/z=480, 482) as well as ions corresponding to loss of deoxyribose and loss of the NPE group. Also, an accurate mass measurement of the molecular ion verified the elemental composition of C₁₈H₁₈O₆N₅⁷⁹Br₁.

Condensation of O⁶-NPE-Protected 2-Bromo-2'-deoxyinosine with Cyclopenta-PAH amino alcohol

Since an O⁶-NPE-protected derivative of 2-bromo-2'-deoxyinosine (9) had been successfully synthesized, it was coupled with enantiomeric trans acenaphthene 1-amino-2-ol

(7) to generate protected N²-deoxyguanosine adducts of a cyclopenta-PAH (10) (see figure 8 for synthetic scheme). With the bulky p-toluoyl groups removed, it was hoped that yields would be improved relative to condensation with the fully protected 2-BrdI, and the resulting adduct product could then be easily transformed into a true deoxyguanosine adduct by removal of the NPE group with DBU.

Procedures for the second condensation reaction were similar to the first. 2-methoxyethanol was used as the solvent for the reflux, but this time argon was passed through the apparatus during the reaction to help prevent oxidation of the cyclopenta-PAH. Work-up of the reaction mixture was carried out on reversed phase prep TLC plates due to the more polar nature of the products.

Ten bands were resolved on the plates, and all products were screened for the acenaphthene-deoxyguanosine adduct by UV-visible spectroscopy and FAB mass spectrometry (in a full scan mode). Mass spectra failed to detect the MH⁺ molecular ion (m/z=585) in any band, but revealed an ion at m/z=584 in one fraction. This fraction (6.5 mg), which corresponded to the slow-migrating band 8 on prep TLC plates (R_f=0.24), fluoresced light blue under long wave UV and showed a UV-visible spectrum similar to that of the acenaphthene-dA adduct. It was decided to purify this promising fraction by reversed phase HPLC and then to repeat spectral analysis.

Major peaks eluting at longer retention times were collected, but the trace showed no clear resolution of the

expected diastereomers. In synthesizing N⁶-adenosine adducts, Bartczak et al. (1989) were able to separate the diastereomeric adduct mixtures by reversed phase HPLC.

Product (3.5 mg) from the HPLC purification showed a promising UV-visible spectrum (see figure 20) with a λ_{max} at 280 nm, similar to the acenaphthene-dA adduct. ¹H NMR spectroscopy on this product (see figure 21) revealed a set of signals with integral ratios suggesting a product containing both a deoxyribose moiety as well as a PAH moiety. The spectrum was not definitive, however, due to impurities, so additional HPLC purification was repeated to give 2.5 mg of product from the major eluting peaks. Again, diastereomers were not resolved.

As shown in figure 22, the latter product's UV-visible spectrum (methanol) showed a wide absorption peak with λ_{max} at 280 and 295 nm. Mass spectrometry on this product again failed to confirm the molecular ion expected for the protected N²-modified deoxyguanosine. As mentioned previously in this report, the technique for obtaining the mass spectrum was probably in error, and it is not possible to repeat the analysis due to lack of any remaining adduct product. The ¹H NMR spectrum (500 MHz, DMSO-d₆) of the twice-purified adduct was also not definitive because of interferences. However, the partial ¹H NMR spectrum in figure 23 shows better resolution of the proton signals in the aromatic region than was observed earlier.

Thus, presence of desired compound 10 could not be confirmed on the basis of the spectra obtained, although some indication of the protected acenaphthene-deoxyguanosine adduct product was seen. If the protected adduct was indeed generated from this condensation reaction, the yield would have been approximately 4% (based upon the weight of the final purified product). Most likely, the very high temperatures ($>130^{\circ}\text{C}$) of the refluxing 2-methoxyethanol contributed to decomposition of the cyclopenta-PAH starting material (7), adversely affecting the reaction yields.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Summary and Conclusions

The objectives of this study were met. During the synthetic reactions, important results were obtained, which provide a basis for continued research as well as recommendations for improvement in future synthetic strategies.

As shown in figures 7 and 8, a straightforward synthetic route, which has not been reported in the literature, was developed for the synthesis of an O⁶-NPE and p-toluoyl group protected derivative of 2-bromo-2'-deoxyinosine (6B). This successful strategy involved sodium salt glycosylation of O⁶-NPE-protected 2-bromohypoxanthine (4) with chlorofuranose (5B) to give desired pure N-9 β glycosylation product (6B) in 39% yield.

Although the three-step synthesis of compound 4 was lengthy and not as straightforward or as efficient as reported (Raju, Robins, and Vaghefi 1989), it could be applied with minor modifications to yield pure 4 in adequate amounts.

An important observation is that the O-6 p-nitrophenylethyl group does not appear to direct the formation of the N-9 β isomer in the sodium salt

glycosylation product as was expected on the basis of literature reports (Raju, Robins, and Vaghefi 1989). The protecting group seems to function primarily to facilitate separation and purification of the isomeric products.

Compound 6B was successfully condensed with trans acenaphthene 1-amino-2-ol (7) to generate the first example of a protected N²-deoxyguanosine adduct of a cyclopenta-PAH (compound 8) in ~5% yield.

The p-toluoyl groups can be removed by ammonolysis of compound 6B to yield O⁶-NPE-protected 2-bromo-2'-deoxyinosine (9) in 63% yield. Again, no procedures have been published for this compound either.

p-Nitrophenylethanol and 2-bromo-2'-deoxyadenosine were identified ammonolysis reaction side-products by spectroscopic analysis. They support the significant finding that the O-6 NPE protecting group can be removed during prolonged reaction, in contradiction to literature reports (Trichtinger, Charubala, and Pfleiderer 1983; Himmelsbach et al. 1984; Aerschot et al. 1988).

As a final step, condensation of compound 9 with the cyclopenta-PAH amino alcohol 7 was carried out, and, although indicated, formation of the desired adduct (compound 10) could not be confirmed in the initial experiment, possibly due to impurities or error in the spectroscopic technique. However, the first successful condensation reaction did prove the usefulness of the protected 2-bromo-2'-deoxyinosine derivative (6B) as a

precursor to target N²-deoxyguanosine adducts of a cyclopenta-PAH (with potential application to other genotoxic chemicals).

In the condensation reactions, the low yields (~4-5%) presumably resulted from decomposition of the CP-PAH amino alcohol (7) due to very high refluxing temperatures. Even though the yields were low, even a few milligrams of synthetic adduct compounds will be sufficient amounts for future application as chromatographic standards in ³²P-postlabeling assays.

In order to generate the completely deprotected N²-deoxyguanosine adduct of a CP-PAH, a final treatment with DBU to remove the O-6 NPE group remains to be accomplished. For use as synthetic standards, N²-modified deoxyguanosine adducts would, in addition, need to be converted to their 3'-phosphorylated derivatives by procedures that were also not tested in this study.

Recommendations for Future Research

The synthesis of O⁶-NPE-protected 2-bromohypoxanthine did not proceed quite as smoothly or successfully as anticipated based on published reports (Raju, Robins, and Vaghefi 1989). Yields were much lower than reported. Also, based on results in this study, the function of the O-6 NPE group in directing glycosylation to the N-9 β isomer is questionable. During future syntheses, these reaction procedures should be examined carefully to attempt to

increase yields to the reported values. Investigation of an alternative O-6 protecting group, such as a benzyl group, might also be considered and may offer a less time-consuming, more successful strategy to controlling regiochemistry of glycosylation.

The effectiveness of the O-6 NPE blocking group as claimed in the report by Raju, Robins, and Vaghefi (1989) could be evaluated by conducting an additional reaction. Lewis acid catalyzed glycosylation of 2-bromohypoxanthine with 1,2,3,5-tetraacetylribose should produce only N-9 β and N-7 β isomeric products. From this reaction, relative amounts of N-9:N-7 isomers could then be compared with the reported ratio of 20:1 for O⁶-NPE-protected 2-bromohypoxanthine in order to determine the magnitude of difference between the two purine starting materials.

Since the ammonolysis reactions appeared to produce interesting side-products, further investigation into the removal of the O⁶-NPE protecting group could be undertaken. Future ammonolysis reactions carried out for over 20 hours on a greater amount of starting material (compound 6B) should lead to sufficient amounts of side-products for satisfactory NMR and mass spectra to confirm identities.

Finally, reaction conditions in future condensation reactions with amine alcohols should be adjusted to optimize yields of the desired N²-modified deoxyguanosine adducts. This could include use of a lower boiling solvent, such as ethanol, to help prevent decomposition of the cyclopenta-PAH

amino alcohol, as well as possible alteration of reaction time.

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